# NOVEL HYDROGEN SULFIDE DETECTION METHODS AND THEIR BIOLOGICAL AND INDUSTRIAL APPLICATIONS

by

Megan Kathleen Thorson

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## STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Megan Kathleen Thorson**

has been approved by the following supervisory committee members:

| | | |
|---|---|---|
| **Amy M. Barrios** | , Chair | **9/2/2014** Date Approved |
| **Dennis Winge** | , Member | **9/2/2014** Date Approved |
| **Eric Schmidt** | , Member | **9/3/2014** Date Approved |
| **Caroline Saouma** | , Member | **9/2/2014** Date Approved |
| **Darrell Davis** | , Member | **9/3/2014** Date Approved |

and by **Darrell Davis**, Chair/Dean of

the Department/College/School of **Medicinal Chemistry**

and by David B. Kieda, Dean of The Graduate School.

# ABSTRACT

Hydrogen sulfide ($H_2S$) is a toxic gas with a distinct rotten-egg odor. While the detrimental affects of $H_2S$ on human health have been known for hundreds of years, it has recently become apparent that low levels of endogenously produced hydrogen sulfide are cytoprotective. In fact hydrogen sulfide is an important signaling molecule in a variety of systems resulting in a large range of physiological effects, from vasodilatory to anti-inflammatory. However, the exact roles played by hydrogen sulfide, its concentrations in serum and tissues, and the enzymes that produce it are still being investigated. It seems that a lack of tools with which to accurately measure *in vivo* concentrations of hydrogen sulfide has stalled progress in the field. Herein we report several novel, fluorescent probes with which to detect endogenous concentrations of hydrogen sulfide both *in vitro* and in biological samples. These probes utilize an arylazide moiety that is selectively reduced by hydrogen sulfide to produce a signal. We show that this signal is concentration-dependent in a linear manner up to 300 μM hydrogen sulfide. We also show that these probes are sensitive down to 200 nM hydrogen sulfide. Additionally, we have applied these probes in assays to study hydrogen sulfide-producing enzymes, cystathionine β–synthase (CBS), cystathionine γ–lysase (CGL), and tryptophan synthase (TS). By discovering selective inhibitors of these enzymes, we can modulate hydrogen sulfide production *in vivo*.

Lastly, we have applied a series of lanthanide-based fluorescent probes to detect hydrogen sulfide in the petrochemical industry. These probes utilize a similar arylazide moiety as an antenna. The lanthanide center can be excited after reaction with $H_2S$ to give a signal with a long lifetime. By delaying the readout, it is possible to see this signal over a highly fluorescent background, such as crude oil. These probes have been used to study the presence of hydrogen sulfide in two sour water samples as well as five samples of crude oil obtained from the Tesoro Corporation.

This dissertation is dedicated to my parents, Sue and Roger Thorson, and my husband, William Youmans. Without their encouragement, love, and support this work would not have been possible.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AdoMet | S-Adenosyl-L-methionine |
| ADT | Anethole dithiolethione |
| AOAA | Aminooxyacetic acid |
| ACC | 7-Amino-4-carbamoylmethylcoumarin |
| AMC | 7-Amino-4-methylcoumarin |
| AzCC | 7-Azido-4-carbamoylmethylcoumarin |
| AzMC | 7-Azido-4-methylcoumarin |
| BME | β-mercaptoethanol |
| CBS | Cystathionine β-Synthase |
| CGL | Cystathionine γ-Lyase |
| DADS | Diallyl disulfide |
| DATS | Diallyl trisulfide |
| DTT | Dithiothreitol |
| GSH | Glutathione |
| HA | Hydroxylamine |
| $H_2S$ | Hydrogen sulfide (implies $H_2S$, $HS^-$, and $S^{2-}$) |
| L-DOPA | L-3,4-dihydroxyphenylalanine |
| NaHS | Sodium hydrogen sulfide |

| | |
|---|---|
| NSAID | Non-steroidal anti-inflammatory drug |
| PLP | Pyridoxal 5’-phosphate |
| SAC | S-Allyl cysteine |
| TCEP | Tris(2-carboxylethyl)phosphine |
| TS | Tryptophan synthase |

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Lastly, I would like to express my most sincere gratitude to those who continue to love and support me: my incredible parents Sue and Roger Thorson, my twin sister Amanda Ferrell, and my loving husband C. William Youmans.

# CHAPTER 1

# INTRODUCTION

## 1.1 Hydrogen Sulfide

Hydrogen sulfide ($H_2S$) is a malodorous gas long known for its potent toxicity. In 1713, a study on the effects of hydrogen sulfide on Italian cesspool workers described inflammation of the eyes and eventual blindness.[1–3] Five decades later, gases rising from the sewers of Paris (later identified as $H_2S$) wreaked havoc among the population. Many people suffered the same optic neuritis as the Italian workers with others facing more severe reactions: asphyxia and death.[2] For over 2 centuries since these incidents, hydrogen sulfide was regarded as a noxious, toxic gas. Therefore, it was surprising when, in 1982, it was discovered that human enzymes are capable of producing $H_2S$.[4] In 1996 it was further suggested that $H_2S$ serves as a neuromodulator in the brain.[5] Since then, there has been an explosion of interest in the potential physiological roles of $H_2S$.

The work described in this dissertation is centered around the development of novel chemical tools for studying enzymatically produced and environmental $H_2S$. In this chapter, we will briefly describe the ambiguous physiological effects of endogenous $H_2S$ and $H_2S$-donating compounds, the probable mechanisms of *in vivo* $H_2S$ production (focusing on the enzymatic pathways), and the current detection methods used to study $H_2S$. We will also briefly discuss the challenges of detecting $H_2S$ in various industrial

processes, with an emphasis on the petrochemical industry. First, we will begin with a brief discussion on the chemistry of sulfur and sulfide.

*1.1.1 Chemistry of sulfur and hydrogen sulfide.* Sulfur, like oxygen, is a chalcogen. However, the two have widely varying chemical reactivities. Because of its six valence electrons and empty 3d orbital, sulfur can take on numerous oxidation states ranging from (-2) at its most reduced to (+6) at its most oxidized. Additionally, sulfur is significantly less electronegative than oxygen, so it does not participate in significant S-H-S hydrogen bonding,[6] making hydrogen sulfide a gas at ambient pressures and temperatures and quite lipophilic when fully protonated.

Hydrogen sulfide is an example of sulfur in its most reduced form. As such, it is nucleophilic, reacting with electrophiles in addition and substitution reactions, reducing disulfide bonds, and coordinating to positively charged metal centers.[6,7] Hydrogen sulfide is also a weak acid. Reported p$K_a$ values for the first deprotonation vary slightly between 6.76 and 7.0, while the second p$K_a$ value can range from 12 to 19 depending on temperature, pressure, and ionic strength (Figure 1.1).[8–12] Under physiological conditions, $H_2S$ exists mostly in the anionic state, $HS^-$, with approximately 20% present as $H_2S$. It should be noted that because of this equilibrium, *$H_2S$* and *hydrogen sulfide* in this dissertation will refer to all three sulfide species. We will not distinguish between protonation states.


Figure 1.1 Dissociation of hydrogen sulfide ($H_2S$). *$H_2S$* in this work will indicate all three species.

## 1.2 Hydrogen Sulfide in Human Health

*1.2.1 Physiological effects of hydrogen sulfide.* Since its recognition as a biologically relevant gas in the 1980s, it has been well accepted that $H_2S$ is produced endogenously in a myriad of organs where it serves numerous biological functions. Though its exact effects are location and concentration dependent, hydrogen sulfide has been found to play a variety of physiological roles; however, these roles are somewhat incongruous. For example at low micromolar concentrations hydrogen sulfide appears to be cytoprotective through upregulation of antioxidant pathways and anti-inflammatory genes.[13–16] At higher concentrations, hydrogen sulfide is cytotoxic most likely because of oxidant generation and glutathione depletion.

In general, cellular hydrogen sulfide is more often reported as a salutary gas than a toxic gas with regard to biology. In section 1.1 we mentioned the discovery of $H_2S$ as a neuromodulator. Additionally, $H_2S$ is neuroprotective in the brain,[17–19] and decreased $H_2S$ levels are associated with neurodegenerative disorders such as Alzheimer's Disease.[20] Hydrogen sulfide serves as an airway relaxant and anti-inflammatory in the lungs where it has been studied in an asthmatic mouse model.[15,21–23] Hydrogen sulfide is also a regulator of insulin in the pancreas[8,24] and is important in mucosal defense and repair in the gastrointestinal tract.[25–28] The various anti-cancer properties of $H_2S$ are still being investigated, though the results look promising, particularly in breast and colorectal cancers.[29–34] One of the most well studied systems with regard to $H_2S$ is the cardiovascular system where $H_2S$ is cytoprotective, vasodilatory, and anti-inflammatory. It has been reported that CGL knockout mice are hypertensive and that spontaneously hypertensive mice have lower plasma sulfide levels. Indeed hydrogen sulfide-releasing

compounds appear to have antihypertensive activity, underscoring the importance of $H_2S$ in blood pressure regulation.[3,7,9,35–37]

Hydrogen sulfide and its related species are clearly important to human health. By elucidating the activities of enzymes capable of producing $H_2S$ and the ways in which $H_2S$ attenuates so many disorders, it may be possible to better understand and treat these conditions. In Chapter 2 of this dissertation, we describe the development of two novel, fluorogenic probes for $H_2S$. These probes have utility in studying $H_2S$-producing enzymes *in vitro* as part of a novel assay as described in Chapter 3. This should allow us to look for small molecules that can attenuate enzyme activity *in vivo.* Additionally, these probes will also be useful in further studying the release of $H_2S$ from small molecule sulfide donors (See Chapter 4, for example).

*1.2.2 Possible reactivity of hydrogen sulfide in vivo.* Hydrogen sulfide has a plethora of cellular targets as indicated by its widespread physiological effects. While its specific molecular targets and pathways are not fully understood and are beyond the scope of this dissertation, several general methods by which $H_2S$ effects cellular processes will be discussed (Figure 1.2).

As mentioned in section 1.1.1, hydrogen sulfide is nucleophilic. It is therefore capable of covalently labeling proteins through addition and substitution reactions. One such reaction is the attack by hydrogen sulfide on the porphyrin ring of oxygenated hemoglobin, incorporating sulfur into the pyrrole ring.[6,38–40] The mechanism and activity of this unique molecule and its myoglobin analog have intrigued researchers since their discoveries in the late 19$^{th}$ century.[38] Another example of the nucleophilicity of hydrogen sulfide is the formation persulfides by direct sulfhydration of protein cysteine residues.[41]

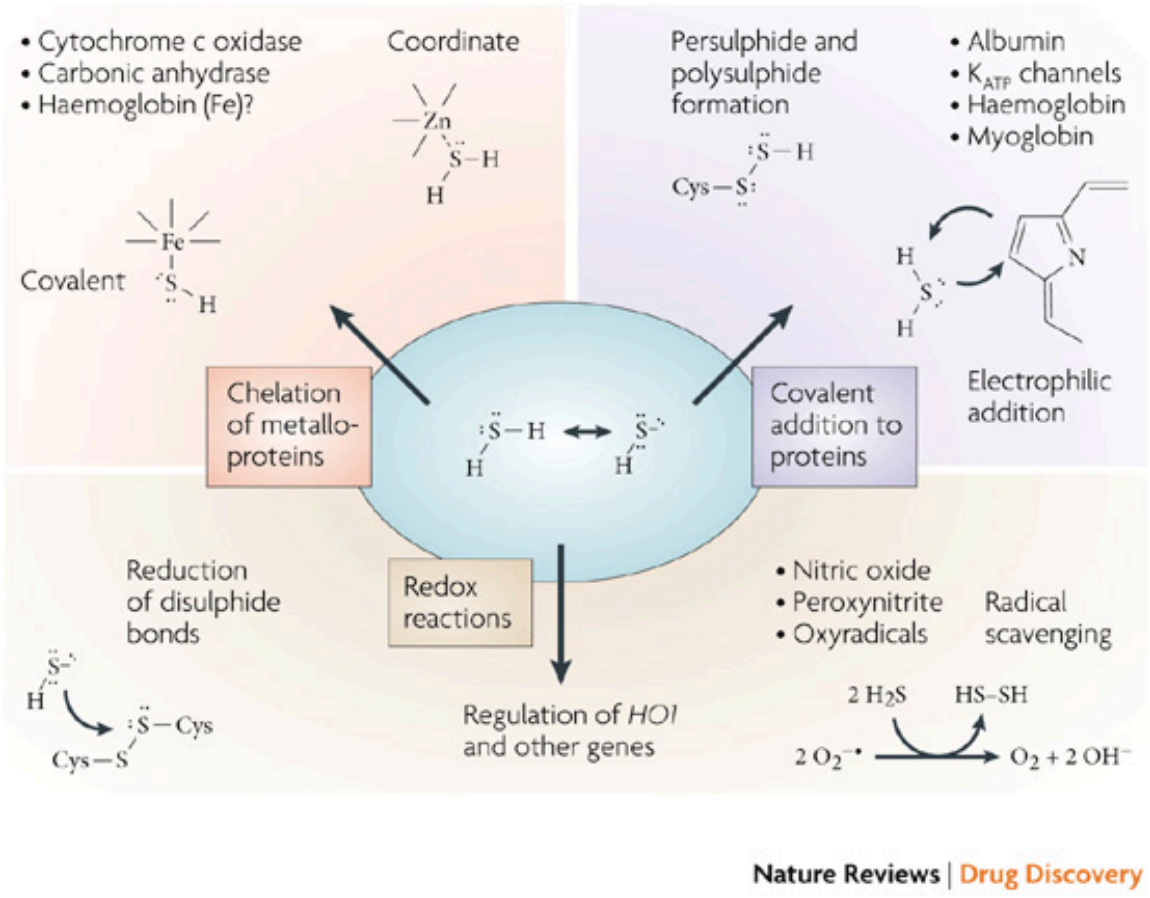


Figure 1.2 Proposed reactivity and cellular targets of hydrogen sulfide. This figure was reprinted with permission from Macmillan Publishers Ltd: Nature Review Drug Discovery (Szabó, C. Hydrogen Sulphide and its Therapeutic Potential. *Nat. Rev. Drug Discov.* **2007**, *6*, 917–935), copyright 2007.

This reaction is analogous to the nitrosylation of proteins by NO, the predominant signaling mechanism for this neuromodulator. In fact, it has been proposed that sulfhydration may be more prevalent than nitrosylation and possibly equal in prevalence to phosphorylation.[41] A recently published study instead suggests that the sulfhydration products of cysteine (thiocysteine) and glutathionine (GSSH) may be the reactive species responsible for the biological effects attributed to hydrogen sulfide.[42,43] Whether these

hydropersulfides are formed directly via human enzymes or are reactive intermediates resulting from a reaction between biological thiols and enzymatically produced hydrogen sulfide remains to be determined. This issue will be further discussed in section 1.2.3.

In addition to the covalent modification of proteins, hydrogen sulfide can also bind to metal centers. Our ability to smell hydrogen sulfide is a result of $H_2S$ binding to copper-activated olfactory receptors.[44,45] Metalloenzymes such as carbonic anhydrase and cytochrome c oxidase may also be inhibited by direct binding of $H_2S$.[46,47]

Lastly, the reducing power of $H_2S$ can be utilized to scavenge reactive oxygen species (ROS) and modify enzymes through the reduction of disulfide bonds. Though the antioxidant ability of $H_2S$ was originally thought to be a major contributor to its anti-inflammatory effects,[48–50] $H_2S$ shows little reactivity towards ROS other than HOCl under cellular conditions.[6,51,52] Therefore, direct radical scavenging by hydrogen sulfide most likely plays a minor role in its reported biological effects. However, one could postulate that persulfide and polysulfide intermediates may have potential reactivity with ROS. Ida et al. suggest that GSSH may have antioxidant potential against hydrogen persoxide[42] though other ROS were not tested, and the relevance of this reactivity *in vivo* is not known. The redox activities of hydrogen sulfide are more easily seen in disulfide bond reduction. It has been proposed that this is the mechanism governing the reversible inhibition of sodium channels by $H_2S$.[53] The reaction between a disulfide and $H_2S$ would result in a free thiol in addition to a persulfide. It is possible that the reaction between a disulfide such as cystine and $H_2S$ would result in thiocysteine, which could then further react with other thiols and disulfides.

*1.2.3 Hydrogen sulfide production in vivo.* Given its importance to human health

and biology, it is to be expected that the endogenous production and cellular manipulation of hydrogen sulfide has been an area of great interest. Despite the intense investigation in this area, the major mechanisms of hydrogen sulfide production and even the major cellular sources of hydrogen sulfide are still not clear. It has been established that endogenous hydrogen sulfide in humans is dependent on three location-dependent enzymatic systems: two pyridoxal 5'-phosphate (PLP)-dependent enzymes, cystathionine β–synthase (CBS) and cystathionine γ–lyase (CGL), and the coupled activities of cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (3-MCT). While the importance of the CAT/3-MCT pathway has recently come to light, our focus will be on the two PLP-dependent enzymes CBS and CGL (Figure 1.3).

Structurally, CBS is a type II-fold, PLP-dependent enzyme consisting of an N-terminal heme, a highly conserved core, and a C-terminal regulatory domain often labeled the *CBS domain* (Figure 1.4).[54–57] Originally studied for its role in homocysteine metabolism, CBS catalyzes the first step in the transulfuration pathway. The principal reaction catalyzed by CBS is thought to be a beta-replacement reaction between serine and homocysteine, producing cystathionine and water in the process (Figure 1.5).[58,59] Replacement of an active site lysine with the amino terminus of an amino acid (serine and cysteine are pictured), deprotonation, and elimination of $H_2X$ result in the formation of an aminoacrylate intermediate.[54,56,60–62] Nucleophilic attack by homocysteine (the rate determining step for this reaction) on this intermediate produces an external aldimine. Elimination of the reaction products and reformation of the Lys-PLP adduct then complete the cycle.[54,56,59,61,62] The catalytic activity of CBS is enhanced up to three-fold through the binding of an allosteric regulator, S-adenosyl-L-methionine (AdoMet), to the

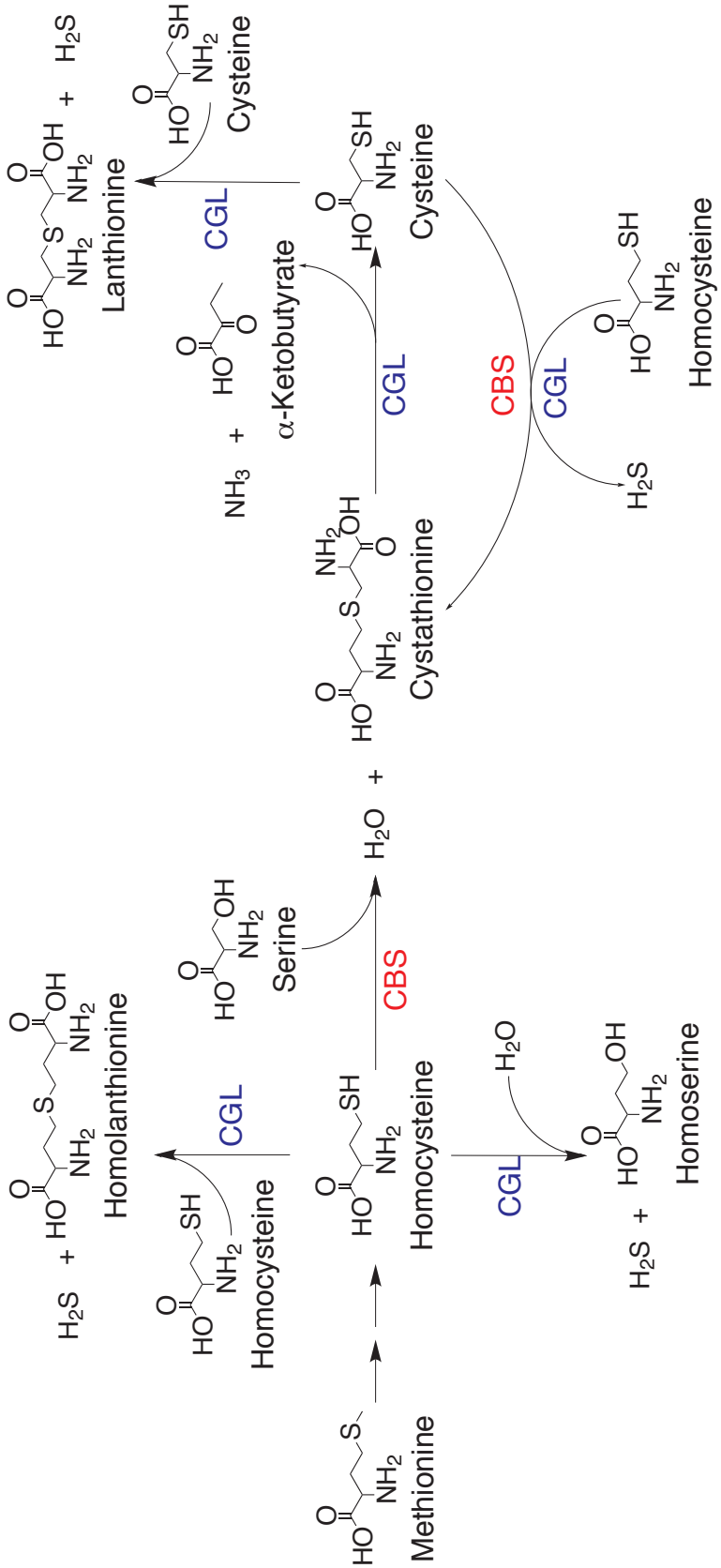


Figure 1.3 Enzymatic production of hydrogen sulfide from CBS and CGL.

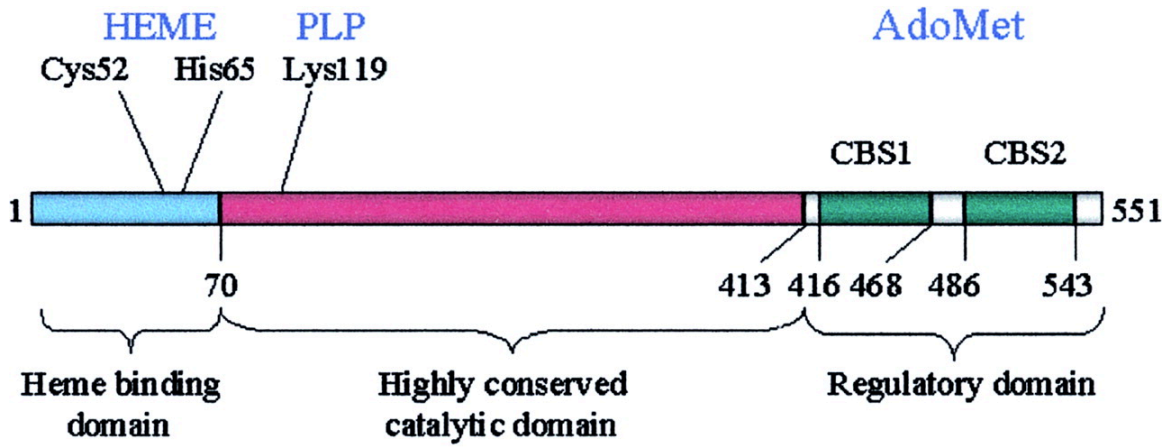


Figure 1.4 Structure of cystathionine β–synthase (CBS) subunit. This figure was originally published in the Journal of Biological Chemistry. Edith W. Miles and Jan P. Kraus. Cystathionine Beta-Synthase: Structure, Function, Regulation, and Location of Homocystinuria-Causing Mutations. *J. Biol. Chem*. 2004; 279: 29871–29874. © The American Society for Biochemistry and Molecular Biology

regulatory domain.[55,63–66] While not clearly understood, it has been proposed that binding of AdoMet induces a conformational change, exposing an active core.[63,65] In mutations lacking this regulatory domain, a highly active enzyme is formed.[58,63]

PLP-dependent enzymes such as CBS appear to be fairly promiscuous. In Chapter 2, we describe the production of hydrogen sulfide from another type II-fold PLP-dependent enzyme, tryptophan synthase (TS). This bacterial enzyme catalyzes the last step in the synthesis of tryptophan. Although TS is not known to naturally produce $H_2S$, TS will catalyze a β-replacement reaction between cysteine and homocysteine under optimized assay conditions. Likewise, it has been reported that CBS is capable of catalyzing a number of β-replacement reactions (Figure 1.6). For example in addition to coupling serine and homocysteine, CBS can also catalyze the reaction between cysteine and serine to form lanthionine. However, this reaction is very slow, proceeding at 0.8%

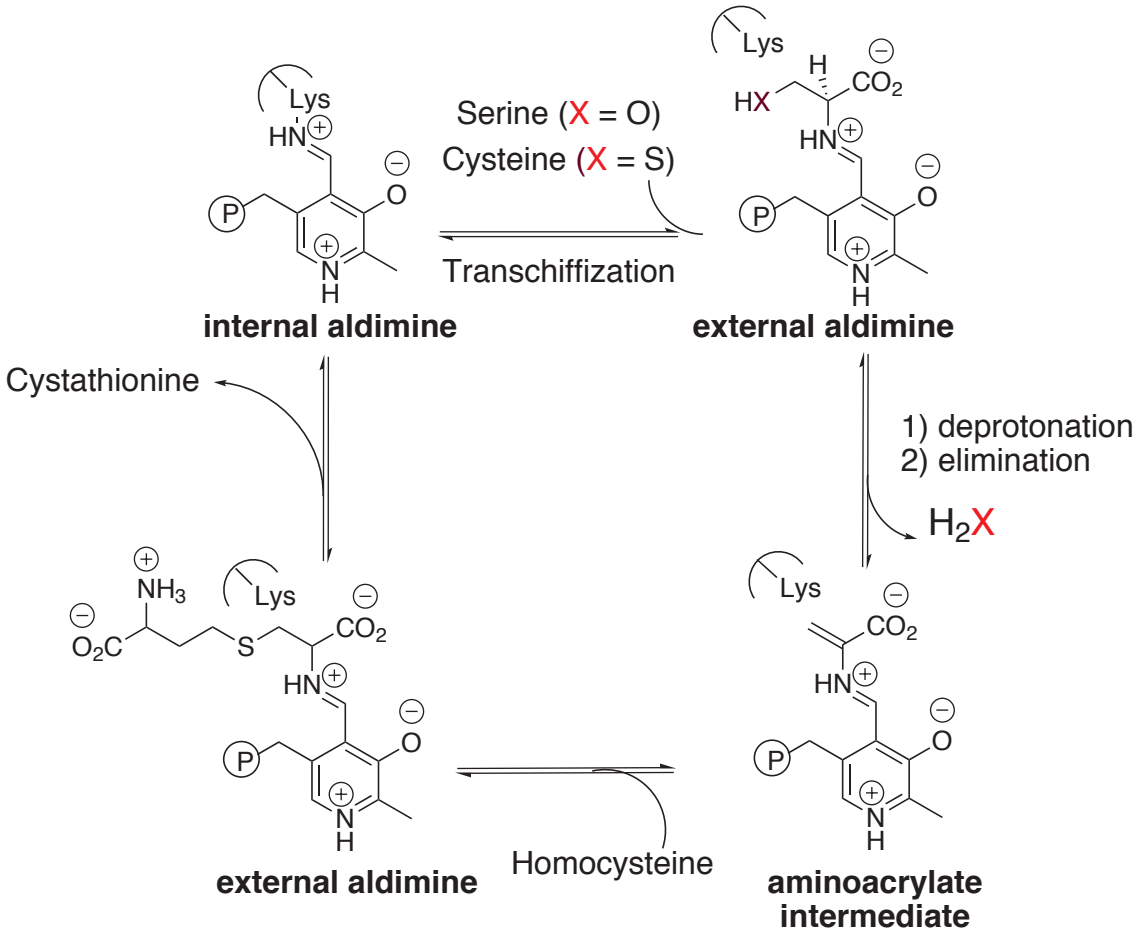


Figure 1.5 Mechanism of homocysteine metabolism by CBS.

that of the more traditional reaction.[61] Additionally, Ida and coworkers reported the CBS-catalyzed conversion of cystine to thiocysteine.[42] Again, only a very small turnover was detected (1% in 30 min). In general, CBS can catalyze a number of β–replacement reactions, but there is little evidence that it is capable of efficiently catalyzing a β–elimination reaction[67] such as that described by Ida and coworkers. In experiments using both yeast extracts containing human CBS and recombinant CBS isolated from *E. coli.,* no elimination of $H_2S$ was detected when cysteine was the only substrate present.[58]

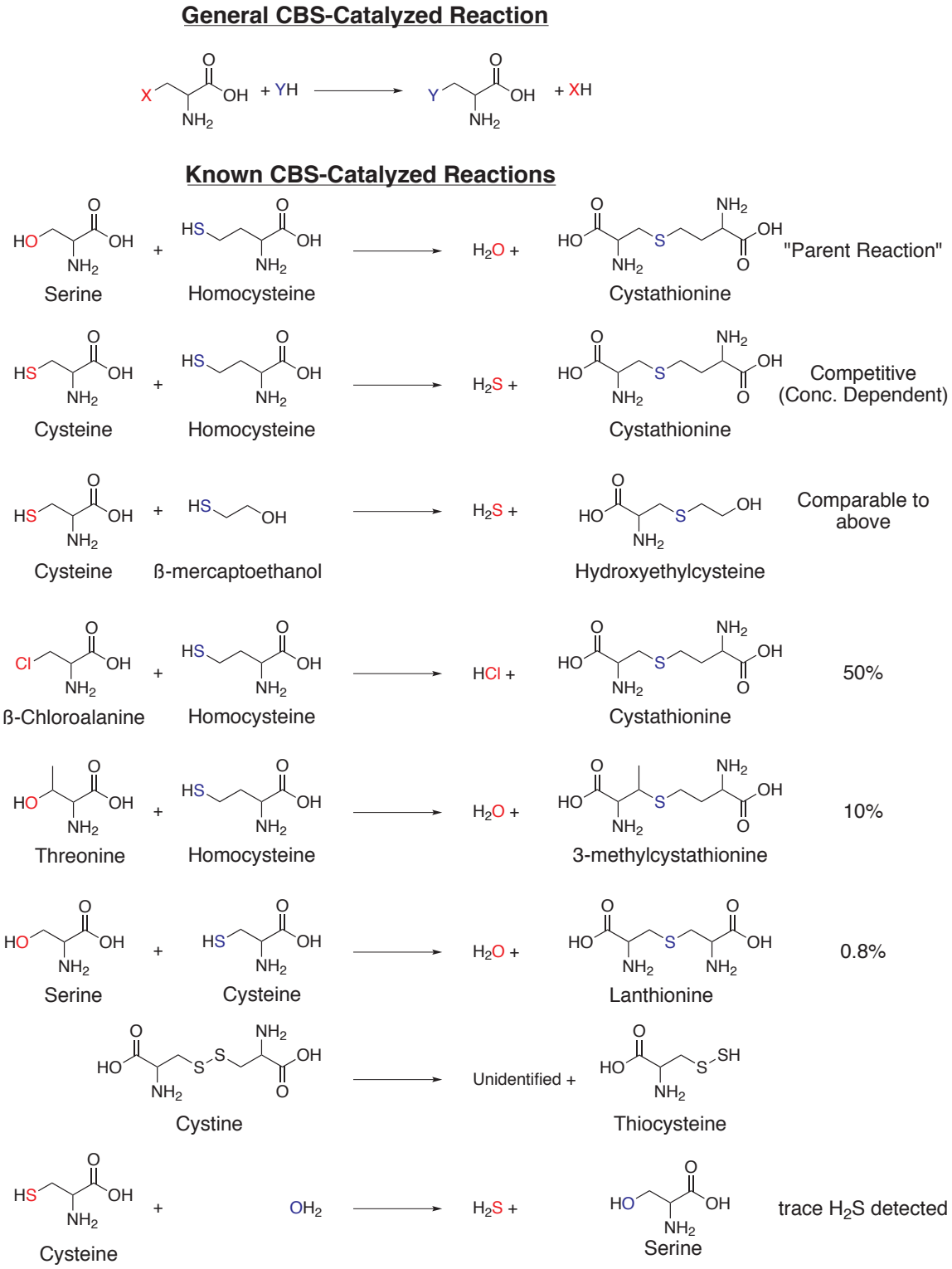


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Figure 1.6 Reactions catalyzed by human CBS. Numbers indicate relative efficiency compared to parent reaction

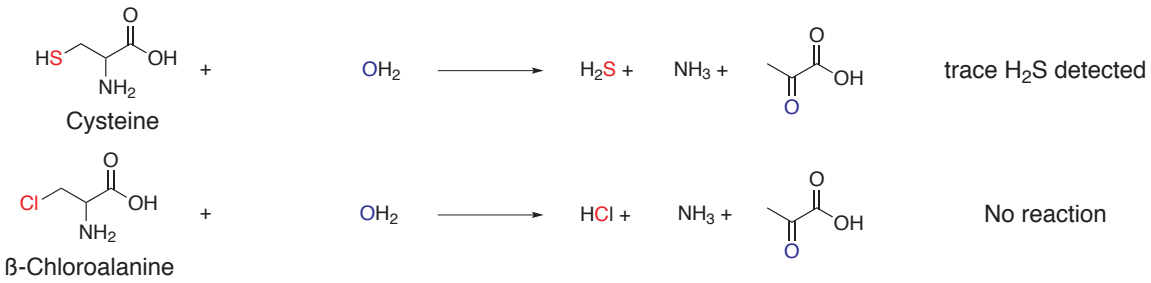



Figure 1.6 continued

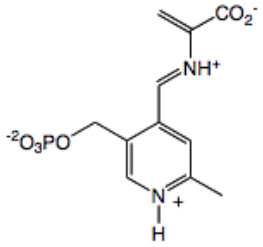
Addition of a thiol such as β-mercaptoethanol (BME) or homocysteine was required. This finding fits with the CBS mechanism previously described. Without nucleophilic attack on the aminoacrylate intermediate to precipitate release of the product, the reaction is not efficient. This may explain the low level of persulfide formation in the presence of cystine alone.[42] The same trend was also seen in our own experiments with tryptophan synthase: the addition of homocysteine greatly improved catalytic turnover over the use of cysteine alone.

It is difficult to speculate which reactions are carried out by CBS and CGL *in vivo*. Most *in vitro* studies on CBS and CGL require a highly reducing environment, while those performed using cystine as the substrate did not. Additionally, it may depend on the localization of the enzyme. Cysteine concentrations were found to be 100-fold higher than cystine in the cytosol of leukocytes and 10-fold higher in fibroblasts.[68] Others have reported the reverse in plasma or extracellular fluid.[69,70] Using competition experiments between cysteine and serine, Chen and coworkers found that preference for the β-replacement reaction catalyzed by CBS was extremely dependent on absolute substrate concentrations.[58] When 10 mM cysteine, serine, and homocysteine were present, the amount of cystathionine derived from cysteine and serine reactions was nearly equal; however, under 0.1 mM substrate concentration conditions, only 23% of the cystathionine produced was a result of cysteine metabolism. Complicating this issue even more are the nonenzymatic reactions possible under these conditions. For example it may be possible for hydrogen sulfide produced by CBS to interact with thiol substrates such as cysteine to form persulfides. Additionally, reports indicating the direct production of thiocysteine from CBS and CGL could instead be a result of a small cysteine contaminant

being converted into hydrogen sulfide, which then reacts with the cystine to form thiocysteine.[19]

With so many variables and so many possible reactions, it can be difficult to study the activity of CBS *in vitro.* Since the reactions catalyzed by CBS go through a common intermediate and often form cystathionine as a common product, interfering with the ability of CBS (or CGL) to catalyze one reaction should inhibit the enzymes' ability to catalyze all reactions. Indeed, the activity of CBS has been traditionally studied using a radioactive serine assay in conjugation with homocysteine (Table 1.1). We have seen that inhibitors of this reaction (hydroxylamine, HA and aminooxyacetic acid, AOAA) also inhibit the production of $H_2S$ from CBS in the presence of cysteine and homocysteine. While a more detailed comparison of inhibitor potencies for various CBS-catalyzed made this endeavor difficult. Additionally, the lack of potent and selective CBS inhibitors

Table 1.1 CBS catalyzed reactions with reported assays.

| Reagents | Intermediate | Products | Assay & Reference |
|---|---|---|---|
| Serine + NaHS |  The reaction of Ser or Cys with the PLP group of CBS results in the formation of this eneimine intermediate after β-elimination of $H_2O$ or $H_2S$.[71] | Cysteine + $H_2O$ | **1.** Colorimetric assay for cysteine[72,73] |
| Serine + homocysteine | | Cystathionine + $H_2O$ | **2.** Ellman's reagent-based colorimetric assay for hCys[74] |
| | | | **3.** Radiometric assay for $^{14}C$ Ser incorporation into cystathionine[75] |
| Cysteine + Homocysteine | | Cystathionine + $H_2S$ | **4.** Colorimetric assay for cysteine[72,73] |
| | | | **5.** Lead acetate based colorimetric assay for $H_2S$[74] |
| | | | **6.** Methylene blue assay for $H_2S$[4,76] |

reactions is needed, the lack of facile assays for this array of β–replacement reactions has has also hampered studies in this area. Once potent (low μM) and selective inhibitors of one CBS-catalyzed reaction have been discovered and thoroughly investigated, a more detailed study of inhibition under different assay conditions will be in order.

As mentioned above, only two compounds are routinely used to chemically inhibit CBS activity, aminooxyacetic acid (AOAA) and hydroxylamine (HA). These compounds target the PLP cofactor of CBS. Therefore, they are not selective for CBS over other PLP-dependent enzymes, namely CGL. Potent, selective inhibitors of CBS activity would thus have great use in this field and could further our knowledge of physiological hydrogen sulfide (either as a free gas or a reactive persulfide intermediate). In Chapter 3, our efforts to identify selective CBS activators and inhibitors via enzyme activity screens are discussed.

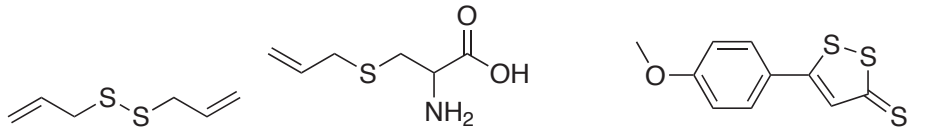
While it is generally accepted that endogenous hydrogen sulfide production is dependent on the three enzymatic pathways just described, it is not clear if this $H_2S$ is immediately released or if it is instead stored until a physiological event triggers its release. Indeed two types of sulfur stores are known: acid-labile sulfur stores and reduction-labile sulfur stores. Acid-labile sulfur pools can be found in mitochondria and most likely take the shape of metal-sulfide clusters.[19,77] However, because mitochondria are not acidic, these pools may or may not have physiological relevance. Therefore, reduction-labile sulfur pools may be the dominant source of stored sulfur in cells. Indeed, Ishigami and coworkers have reported that exogenously applied $Na_2S$ was readily absorbed by heart, liver, and brain homogenates and that this increased reduction-labile sulfur stores without affecting the quantity of acid-labile sulfur.[18] Additionally, it has

been hypothesized that persulfides and polysulfides may serve as the source of this reduction-labile sulfur.[78–89] Low molecular weight persulfides have biological importance. They are capable of easily transferring a terminal sulfur to protein thiols such as cysteine to form new persulfides. This transfer may be more readily controlled than free sulfide ($H_2S$). Additionally, these newly modified proteins often exhibit altered activity from their unmodified forms (that is, persulfide modifications can often activate or inhibit enzyme activity) or these proteins can be "carrier proteins" further modifying tRNA or serving as sulfur sources for the synthesis of sulfur-containing cofactors and vitamins. As a chemical model for persulfide reactivity, Bailey and coworkers synthesized and fully characterized the hydropersulfide tritylhydrodisulfide (TrtSSH) and found that TrtSSH could be reduced in organic solvent to from the thiol TrtSH and sulfide.[90] Thus it is possible that, rather than immediately reacting, endogenously produced $H_2S$ could be oxidized into persulfide or polysulfide complexes that can either react directly with protein targets[42] or can be rereduced to liberate hydrogen sulfide when needed.[18] Another hypothesis is that, as mentioned above, the $H_2S$-producing enzymes CBS and CGL catalyze the direct synthesize of persulfides (from sources such as cystine) in addition to free $H_2S$ (from cysteine and homocysteine). These persulfides could then either react with molecular targets, transferring a terminal sulfur in the process, or they could release $H_2S$ under reducing conditions. It is not clear which reactive sulfur species is produced most abundantly *in vivo* nor which species is responsible for the reported biological effects of $H_2S$: persulfides, hydrogen sulfide, acid-labile sulfur pools, or an as-yet undiscovered sulfide containing species. It is also yet to be determined which, if any, of the $H_2S$-detection methods (described in more detail in section 1.3 and Chapter 2) are

selective for unbound $H_2S$ over persulfide complexes.

*1.2.4 $H_2S$-donating compounds.* Because of the interest in understanding endogenous hydrogen sulfide and activated sulfur compounds, a variety of small molecules capable of modifying $H_2S$ levels have been identified. These compounds typically use one of two methods: some compounds affect $H_2S$-producing enzyme activity as just discussed while others are metabolized to release $H_2S$ directly. Many of these *$H_2S$-donating compounds* can be found in nature. For example, the health benefits of garlic consumption have long been known.[91–95] Is has been postulated that these antioxidatant and cardioprotective effects are a result of $H_2S$-release from a garlic metabolite, diallyl disulfide (DADS) (Figure 1.7), which produces $H_2S$ in the presence of glutathione.[10,32,34,96] It is also possible that DADS could produce an active GSSH complex that is detected as $H_2S$ in reducing environments. Another $H_2S$-donating compound found in garlic, S-allyl cysteine (SAC), has inspired a number of derivatives. These compounds are metabolized by CGL to form $H_2S$ and appear to greatly effect the cardiovascular system.[7,34,96,97]

In addition to these naturally occurring compounds, synthetic $H_2S$-donating agents are also known. One of the most promising (and therefore well studied) is anethole dithiolethione, ADT (Figure 1.7). ADT has been conjugated to known therapeutics, often NSAIDs, through an ether linkage.[11,15,27,96] The underlying hypothesis behind this




**diallyl disulfide** **S-allyl cysteine** **anethole dithiolethione**

Figure 1.7 $H_2S$-donating compounds.

approach is that the $H_2S$ release will attenuate the gastrointestinal tract inflammation and damage caused by routine consumption of these drugs.

$H_2S$-donating compounds show promise as potential therapeutics and will aid in elucidating the roles of $H_2S$ in human health; however, more studies need to be done on these compounds. The mechanism and especially rate of $H_2S$ release for many of these compounds is not well understood. It has been suggested by some that several of these compounds may have biological activity independent of $H_2S$ release. In Chapter 4, we discuss a new $H_2S$-donating compound and compare its reactivity and rate of $H_2S$ release to two known $H_2S$-producing agents.

## 1.3 Current Detection Methods for Hydrogen Sulfide

Hydrogen sulfide is clearly an important molecule in a variety of settings; however, a lack of suitable detection methods has slowed the exploration of this field. For example, controversial endogenous $H_2S$ concentrations in plasma and tissues are most likely a result of insufficient or interfering $H_2S$-detection techniques.

*1.3.1 Traditional detection methods.* Other than the human nose, which loses its ability to detect $H_2S$ concentrations at 50–100 ppm,[9] the oldest detection method for hydrogen sulfide dates back to the 19$^{th}$ century. The methylene blue assay (Figure 1.8) couples two equivalents of 4-aminodimethylaniline and sulfide in the presence of zinc, ferric chloride, and strong acid to produce an aromatic compound with a signature blue color.[76] While this reaction is somewhat selective for $H_2S$ (in our hands a pink color forms in the presence of some thiols such as β–mercaptoethanol, BME), it may not be ideal for biological samples. The presence of cellular acid-labile sulfur pools has been

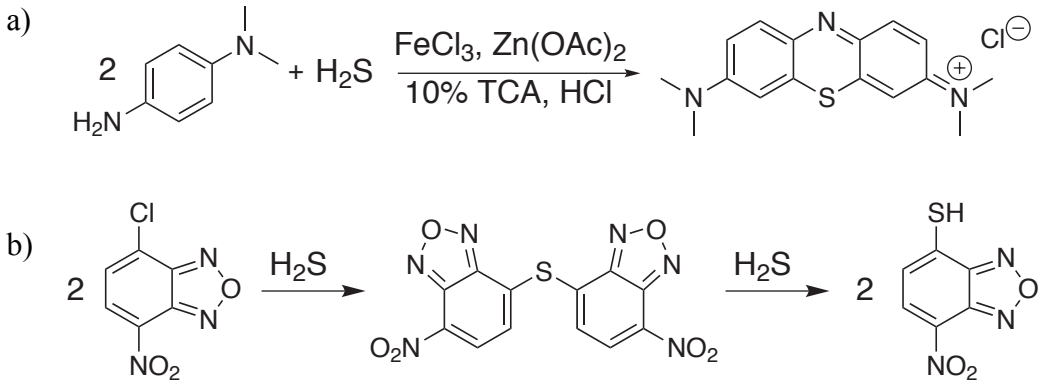



Figure 1.8 Colorimetric hydrogen sulfide detection methods. a) methylene blue b) benzoxadiazole-based assay

reported;[3,10,11,15,18,62,97] these pools are likely to release sulfide under the methylene blue assay conditions, giving a higher experimental concentration than actually exists. Additionally, these conditions could cause sulfide release from enzyme cofactors and other stable sulfur sources. It may be surprising then that methylene blue appears to be the assay of choice for many biologists.

The frequently used standard in the field for the petrochemical and water treatment industries is even more rudimentary. In these fields, the presence of sulfide is determined qualitatively using lead acetate. The presence of lead sulfide as a precipitant is indicative of $H_2S$. Additionally, gas chromatography[98] and the sulfide ion selective electrode[99] are also employed.

*1.3.2 Colorimetric detection methods.* Aside from the methylene blue assay, few colorimetric tests for $H_2S$ are readily available. These assays should have the advantage that they provide an easy readout without the need for costly and bulky equipment; however, they are often not as sensitive as their fluorogenic counterparts. A novel colorimetric assay for $H_2S$ has recently been developed. This assay uses yellow

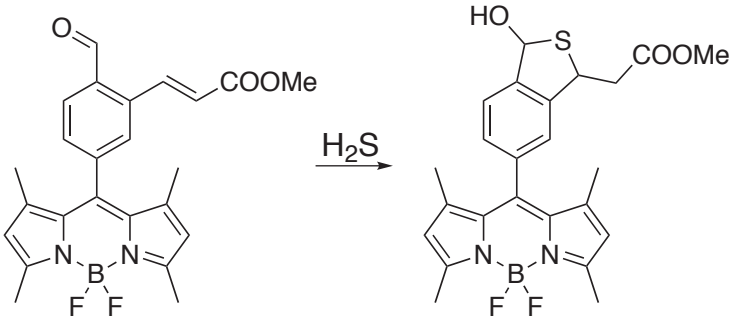
benzoxadiazole-derivates as the $H_2S$ sensor (Figure 1.8). Upon nucleophilic substitution by $H_2S$, the solution becomes pink. The mechanism of this probe is somewhat limiting. An excess of $H_2S$ is required to form the pink product since an intermediate is formed when less than one equivalent is added.

*1.3.3 Fluorescent detection methods.* Thanks to the intense interest in studying physiological hydrogen sulfide, a number of fluorescent probes were developed in the last few years.[100–105] These probes frequently fall into one of several categories: probes that are reduced by $H_2S$, probes that undergo nucleophilic attack by $H_2S$, and metal-based probes.

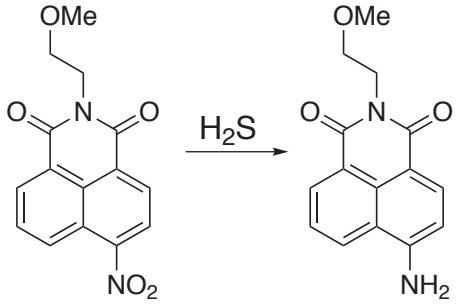
In 2011, the He lab developed a novel fluorescent probe containing two proximal electrophilic moieties (Figure 1.9).[106] This allows for some selectivity as competing thiols can react with one but not both groups. However, this also means that these thiols can reversibly inhibit hydrogen sulfide from binding, reducing the signal in high thiol backgrounds.

Another commonly employed motif in the detection of $H_2S$ has been the use of aryl nitrites and azides. Hydrogen sulfide selectively reduces these moieties to form the amine derivatives. For example, the Pluth group developed nitrite (HSN1) and azide (HSN2) derivatives of amino-naphthaline (Figure 1.9).[104] While HSN1 and HSN2 possess little background fluorescence, the amine product, which forms upon reaction with $H_2S$, is highly fluorescent. As the reduction of nitrite groups is not as selective, HSN1 has a large background in the presence of two biologically relevant thiols, glutathione and cysteine. These probes are also limited to $H_2S$ concentrations greater than 5 μM, their detection limit.

a)




b)




**HSN1**

c)

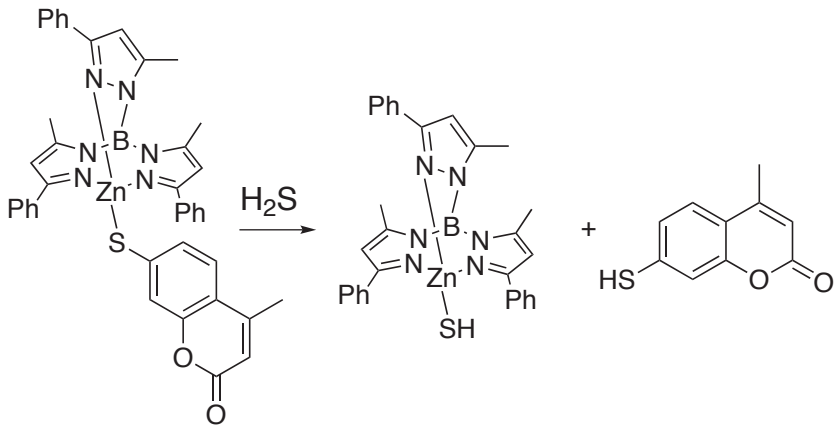



Figure 1.9 Fluorescent $H_2S$-selective probes. a) An example of a probe that undergoes nucleophilic attack by $H_2S$. b) Nitrite and azide probes are reduced by $H_2S$ to form amines. c) A metal based probe eliminates a 7-mercapto-4-methylcoumarin in the presence of $H_2S$.

Lastly, thiophillic metals have also been used as tools to detect $H_2S$. The Galardon lab developed a zinc-based sensor utilizing a coumarin scaffold in 2009 (Figure 1.9).[107] Upon exposure to $H_2S$, the coumarin is displaced, theoretically resulting in a fluorescent signal. Curiously, this is a "turn-off" sensor with the fluorescence diminishing with increasing $H_2S$. A maximum detection limit of 80 μM is seen before the signal completely diminishes.

For utility in biological systems and applications, a fluorescent $H_2S$-selective probe should possess several properties. It should be selective for $H_2S$ over other thiols, reducing agents, and enzyme cofactors; it should give a quick, "turn-on" response to detect $H_2S$ in real time; it should have a large detection range; and it should be sensitive to low levels ( $< 1$ μM ) of $H_2S$. We have developed novel probes to detect $H_2S$ under physiologically relevant conditions, as described in Chapter 2. In addition, fluorogenic and colorimetric probes compatible with industrial applications are described in Chapter 5.

**1.4 Hydrogen Sulfide as an Environmental Pollutant**

In addition to its potential roles in biology and medicine, hydrogen sulfide is also a major player in various industries such as tanneries, paper milling, water treatment, and oil refining. In the latter industry, especially, hydrogen sulfide poses a major challenge. $H_2S$, soluble in both oil and water, must be identified, removed, and processed.

Crude oil is formed by the putrefaction of algae and zooplankton at high temperatures (65–150 ˚C) and pressures over long periods of time (~10 million years).[108] Because sulfur-containing amino acids and biological molecules are broken down,

hydrogen sulfide is often formed. If a crude oil sample contains low sulfide levels (>0.42%) the oil is deemed *sweet*; however, if higher levels of sulfides are found, the oil is deemed *sour*. During the processing of oil, the oil goes through a number of processes (distillation, desalting, alkylation, hydrocracking, etc.) where it comes into contact with water. This eventually removes the hydrogen sulfide from the oil, but creates problematic *sour water.*[109] With exceptionally high levels of $H_2S$, $NH_3$, aromatics, and other contaminants, sour water must be purified before it can be released into the environment or reused in the refinery. This is accomplished by running the sour water through a stripper which strips out all the contaminants. The water is then reused in the factory while the sulfur is often sold as sulfur cakes or further oxidized to form sulfuric acid.

Because hydrogen sulfide is corrosive and an environmental toxin, the Environmental Protection Agency (EPA) has set limits on the concentration of $H_2S$ that can be released in water or sold in gasoline. Hydrogen sulfide in wastewater must be below the subjective olfactory detection limit, while that in oil is regulated to no more than 15 ppm.[110] Therefore, the monitoring of $H_2S$ throughout the oil refining processing is of great importance. With highly fluorescent hydrocarbon contaminants and dark oils, traditional fluorescent and colorimetric detection methods often fail; however, in Chapter 5 we discuss a series of lanthanide-based probes capable of detecting $H_2S$ in high background environments.

### 1.5 Concluding Remarks

Over the last 4 decades, our perception of hydrogen sulfide has shifted from viewing it as a toxic gas and environmental hazard to an essential signaling molecule.

While much is known about its broad physiological effects, much remains to be learned about its exact mechanisms of action and potential reactive intermediates. Selective $H_2S$ detection methods compatible with biological systems and industrial samples would greatly aid in this endeavor. Additionally, selective chemical probes with which to mediate $H_2S$-producing enzyme activity would allow for further investigations into the specific roles of CBS and CGL *in vivo*. In this work, we have focused on developing new chemical tools with which to better study hydrogen sulfide and the enzymes capable of producing it.

### 1.6 References


(1) Ramazzini, B. *Am. J. Public Health* **1713**, *91*, 1380–1382.

(2) Smith, R. P. *Am. Sci.* **2010**, *98*, 1–6.

(3) Polhemus, D. J.; Lefer, D. J. *Circ. Res.* **2014**, *114*, 730–737.

(4) Stipanuk, M. H.; Beck, P. W. *Biochem. J.* **1982**, *206*, 267–277.

(5) Abe, K.; Kimura, H. *J. Neurosci.* **1996**, *16*, 1066–1071.

(6) Nagy, P.; Pálinkás, Z.; Nagy, A.; Budai, B.; Tóth, I.; Vasas, A. *Biochim. Biophys. Acta* **2014**, *1840*, 876–891.

(7) Szabó, C. *Nat. Rev. Drug Discov.* **2007**, *6*, 917–935.

(8) Li, L.; Moore, P. K. *Trends Pharmacol. Sci.* **2008**, *29*, 84–90.

(9) Hughes, M. N.; Centelles, M. N.; Moore, K. P. *Free Radic. Biol. Med.* **2009**, *47*, 1346–1353.

(10) Kolluru, G. K.; Shen, X.; Bir, S. C.; Kevil, C. G. *Nitric Oxide* **2013**, *35*, 5–20.

(11) Whiteman, M.; Le Trionnaire, S.; Chopra, M.; Fox, B.; Whatmore, J. *Clin. Sci.* **2011**, *121*, 459–488.

(12) Singh, S.; Madzelan, P.; Banerjee, R. *Nat. Prod. Rep.* **2007**, *24*, 631–639.

(13) Kimura, H. *Antioxid. Redox Signal.* **2014**, *20*, 783–793.

(14) Gallyas, F. *Br. J. Pharmacol.* **2012**, *166*, 2228–2230.

(15) Olson, K. R. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2011**, *301*, 297–312.

(16) Sen, U.; Pushpakumar, S. B.; Amin, M. A.; Tyagi, S. C. *Nitric Oxide* **2014**, In Press.

(17) Régnier, V.; Billard, J.-M.; Gupta, S.; Potier, B.; Woerner, S.; Paly, E.; Ledru, A.; David, S.; Luilier, S.; Bizot, J.-C.; Vacano, G.; Kraus, J. P.; Patterson, D.; Kruger, W. D.; Delabar, J. M.; London, J. *PLoS One* **2012**, *7*, e29056.

(18) Ishigami, M.; Hiraki, K.; Umemura, K.; Ogasawara, Y.; Ishii, K.; Kimura, H. *Antioxid. Redox Signal.* **2009**, *11*, 205–214.

(19) Kabil, O.; Motl, N.; Banerjee, R. *Biochim. Biophys. Acta* **2014**, *1844*, 1355–1366.

(20) Eto, K.; Asada, T.; Arima, K.; Makifuchi, T.; Kimura, H. *Biochem. Biophys. Res. Commun.* **2002**, *293*, 1485–1488.

(21) Chen, Y. H.; Wu, R.; Geng, B.; Qi, Y. F.; Wang, P. P.; Yao, W. Z.; Tang, C. S. *Cytokine* **2009**, *45*, 117–123.

(22) Perry, M. M.; Hui, C. K.; Whiteman, M.; Wood, M. W.; Adcock, I.; Kirkham, P.; Michaeloudes, C.; Chung, K. F. *Am. J. Respir. Cell Mol. Biol.* **2011**, *45*, 746–752.

(23) Rashid, S.; Heer, J. K.; Garle, M. J.; Alexander, S. P. H.; Roberts, R. E. *Br. J. Pharmacol.* **2013**, *168*, 1902–1910.

(24) Yang, W.; Yang, G.; Jia, X.; Wu, L.; Wang, R. *J. Physiol.* **2005**, *569*, 519–531.

(25) Chan, M. V; Wallace, J. L. *Am. J. Gastrointest. Liver Physiol.* **2013**, 467–473.

(26) Wallace, J. L. *Trends Pharmacol. Sci.* **2007**, *28*, 501–505.

(27) Wallace, J. L. *Antioxid. Redox Signal.* **2010**, *12*, 1125–1133.

(28) Teague, B.; Asiedu, S.; Moore, P. K. *Br. J. Pharmacol.* **2002**, *137*, 139–145.

(29) Chattopadhyay, M.; Nath, N.; Kodela, R.; Sobocki, T.; Metkar, S.; Gan, Z. Y.; Kashfi, K. *Leuk. Res.* **2013**, *37*, 1302–1308.

(30) Chattopadhyay, M.; Kodela, R.; Nath, N.; Dastagirzada, Y. M.; Velziquez-Martinez, C. A.; Boring, D.; Kashfi, K. *Biochem. Pharmacol.* **2012**, *83*, 715–722.

(31) Chattopadhyay, M.; Kodela, R.; Nath, N.; Barsegian, A.; Boring, D.; Kashfi, K. *Biochem. Pharmacol.* **2012**, *83*, 723–732.

(32) Kashfi, K. *Antioxid. Redox Signal.* **2014**, *20*, 831–846.

(33) El-Sayed, A. S.; Shindia, A. A. In *Targets in Gene Thearpy*; **2010**; 119–146.

(34) Powolny, A. A.; Singh, S. V. *Cancer Lett.* **2008**, *269*, 305–314.

(35) Kabil, O.; Banerjee, R. *J. Biol. Chem.* **2010**, *285*, 21903–21907.

(36) Benavides, G. a; Squadrito, G. L.; Mills, R. W.; Patel, H. D.; Isbell, T. S.; Patel, R. P.; Darley-Usmar, V. M.; Doeller, J. E.; Kraus, D. W. *Proc. Natl. Acad. Sci.* **2007**, *104*, 17977–17982.

(37) Li, L.; Rose, P.; Moore, P. K. *Annu. Rev. Pharmacol. Toxicol.* **2011**, *51*, 169–187.

(38) Berzofsky, J. A.; Peisach, J.; Blumberg, W. E. *J. Biol. Chem.* **1971**, *246*, 7366–7372.

(39) Rios-Gonzalez, B. B.; Roman-Morales, E. M.; Pietri, R.; Lopez-Garriga, J. *J. Inorg. Biochem.* **2014**, *133*, 78–86.

(40) Bondoc, L. L.; Chau, M. H.; Price, M. A.; Timkovich, R. *Biochemistry* **1986**, *25*, 8458–8466.

(41) Paul, B. D.; Snyder, S. H. *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 499–507.

(42) Ida, T.; Sawa, T.; Ihara, H.; Tsuchiya, Y.; Watanabe, Y.; Kumagai, Y.; Suematsu, M.; Motohashi, H.; Fujii, S.; Matsunaga, T.; Yamamoto, M.; Ono, K.; Devarie-Baez, N. O.; Xian, M.; Fukuto, J. M.; Akaike, T. *Proc. Natl. Acad. Sci.* **2014**, *111*, 7606–7611.

(43) Miranda, K. M.; Wink, D. a. *Proc. Natl. Acad. Sci.* **2014**, *111*, 7505–7506.

(44) Duan, X.; Block, E.; Li, Z.; Connelly, T.; Zhang, J.; Huang, Z.; Su, X.; Pan, Y.; Wu, L.; Chi, Q.; Thomas, S.; Zhang, S.; Ma, M.; Matsunami, H.; Chen, G.-Q.; Zhuang, H. *Proc. Natl. Acad. Sci.* **2012**, *109*, 3492–3497.

(45) Block, E.; Zhuang, H. In *Biochalcogen Chemistry: The Biological Chemistry of Sulfur, Selenium, and Tellurium*; Bayse, C., Ed.; American Chemical Society: Washington, D.C., **2013**; 1–14.

(46) Innocenti, A.; Zimmerman, S.; Ferry, J. G.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4563–4567.

(47) Laggner, H.; Hermann, M.; Esterbauer, H.; Mueller, M. K.; Exner, M.; Gmeiner, B.; Kapiotis, S. *J. Hypertens.* **2007**, *25*, 2100–2104.

(48) Fu, Z.; Liu, X.; Geng, B.; Fang, L.; Tang, C. *Life Sci.* **2008**, *82*, 1196–1202.

(49) Jha, S.; Calvert, J. W.; Duranski, M. R.; Ramachandran, A.; Lefer, D. J. *Am. J. Physiol. Heart Circ. Physiol.* **2008**, *295*, H801–H806.

(50) Kimura, Y.; Kimura, H. *FASEB J.* **2004**, *18*, 1165–1167.

(51) Nagy, P.; Winterbourn, C. C. *Chem. Res. Toxicol.* **2010**, *23*, 1541–1543.

(52) Carballal, S.; Trujillo, M.; Cuevasanta, E.; Bartesaghi, S.; Möller, M. N.; Folkes, L. K.; García-Bereguiaín, M. A.; Gutiérrez-Merino, C.; Wardman, P.; Denicola, A.; Radi, R.; Alvarez, B. *Free Radic. Biol. Med.* **2011**, *50*, 196–205.

(53) Wang, R. *FASEB J.* **2002**, *16*, 1792–1798.

(54) Bharath, S. R.; Bisht, S.; Harijan, R. K.; Savithri, H. S.; Murthy, M. R. N. *PLoS One* **2012**, *7*, e36267.

(55) Miles, E. W.; Kraus, J. P. *J. Biol. Chem.* **2004**, *279*, 29871–29874.

(56) Aitken, S. M.; Lodha, P. H.; Morneau, D. J. K. *Biochim. Biophys. Acta* **2011**, *1814*, 1511–1517.

(57) Meier, M.; Oliveriusova, J.; Kraus, J. P.; Burkhard, P. *Biochim. Biophys. Acta* **2003**, *1647*, 206–213.

(58) Chen, X.; Jhee, K.-H.; Kruger, W. D. *J. Biol. Chem.* **2004**, *279*, 52082–52086.

(59) Banerjee, R.; Evande, R.; Kabil, Ö.; Ojha, S.; Taoka, S. *Biochim. Biophys. Acta* **2003**, *1647*, 30–35.

(60) Dunn, M. F.; Niks, D.; Ngo, H.; Barends, T. R. M.; Schlichting, I. *Trends Biochem. Sci.* **2008**, *33*, 254–264.

(61) Banerjee, R.; Zou, C.-G. *Arch. Biochem. Biophys.* **2005**, *433*, 144–156.

(62) Singh, S.; Banerjee, R. *Biochim. Biophys. Acta* **2011**, *1814*, 1518–1527.

(63) Janosík, M.; Kery, V.; Gaustadnes, M.; Maclean, K. N.; Kraus, J. P. *Biochemistry* **2001**, *40*, 10625–10633.

(64) Baykov, A. a; Tuominen, H. K.; Lahti, R. *Chem. Biol.* **2011**, *6*, 1156–1163.

(65) Hnízda, A.; Spiwok, V.; Jurga, V.; Kozich, V.; Kodícek, M.; Kraus, J. P. *Biochemistry* **2010**, *49*, 10526–10534.

(66) Yadav, P. K.; Xie, P.; Banerjee, R. *J. Biol. Chem.* **2012**, *287*, 37611–37620.

(67) Borcsok, E.; Abeles, R. H. *Arch. Biochem. Biophys.* **1982**, *213*, 695–707.

(68) De Graaf-Hess, A.; Trijbels, F.; Blom, H. *Clin. Chem.* **1999**, *45*, 2224–2228.

(69) Banjac, A.; Perisic, T.; Sato, H.; Seiler, A.; Bannai, S.; Weiss, N.; Kölle, P.; Tschoep, K.; Issels, R. D.; Daniel, P. T.; Conrad, M.; Bornkamm, G. W. *Oncogene* **2008**, *27*, 1618–1628.

(70) Jones, D. P.; Mody Jr., V. C.; Carlson, J. L.; Lynn, M. J.; Sternberg Jr., P. *Free Radic. Biol. Med.* **2002**, *33*, 1290–1300.

(71) Toney, M. D. *Biochim. Biophys. Acta* **2011**, *1814*, 1407–1418.

(72) Gaitonde, M. K. *Biochem. J.* **1967**, *104*, 627–633.

(73) Frank, N.; Kent, J. O.; Meier, M.; Kraus, J. P. *Arch. Biochem. Biophys.* **2008**, *470*, 64–72.

(74) Chiku, T.; Padovani, D.; Zhu, W.; Singh, S.; Vitvitsky, V.; Banerjee, R. *J. Biol. Chem.* **2009**, *284*, 11601–11612.

(75) Kraus, J. P. *Sulfur and Sulfur Amino Acids*; Methods in Enzymology; Elsevier, 1987; Vol. 143, pp. 388–394.

(76) Fischer, E. *Berichte der Dtsch. Chem. Gesellschaft* **1883**, *16*, 2234–2236.

(77) Olson, K. R. *Biochim. Biophys. Acta - Bioenerg.* **2009**, *1787*, 856–863.

(78) Toohey, J. I. *Anal. Biochem.* **2011**, *413*, 1–7.

(79) Francoleon, N. E.; Carrington, S. J.; Fukuto, J. M. *Arch. Biochem. Biophys.* **2011**, *516*, 146–153.

(80) Fukuto, J. M.; Carrington, S. J.; Tantillo, D. J.; Harrison, J. G.; Ignarro, L. J.; Freeman, B. A.; Chen, A.; Wink, D. A. *Chem. Res. Toxicol.* **2012**, *25*, 769–793.

(81) Beinert, H. *Eur. J. Biochem.* **2000**, *267*, 5657–5664.

(82) Mueller, E. G. *Nat. Chem. Biol.* **2006**, *2*, 185–194.

(83) Toohey, J. I. *Biochem. Cell Biol.* **1986**, *64*, 758–765.

(84) Toohey, J. I. *Biochem. J.* **1989**, *264*, 625–632.

(85) Kessler, D. *FEMS Microbiol. Rev.* **2006**, *30*, 825–840.

(86) Dagis, A. I.; Vitkiavichius, K. T.; Bal'chiunas, G. A.; Gendvilene, V. I.; Dzheia, P. P.; Toleĭkis, A. I. *Biull. Eksp. Biol. Med.* **1990**, *110*, 377–379.

(87) Cerletti, P. *Trends Biochem. Sci.* **1986**, *11*, 369–372.

(88) Lang, T.; Kessler, D. *J. Biol. Chem.* **1999**, *274*, 189–195.

(89) Flavin, M. *J. Biol. Chem.* **1962**, *237*, 768–777.

(90) Bailey, T. S.; Zakharov, L. N.; Pluth, M. D. *J. Am. Chem. Soc.* **2014**, *136*, 10573–10576.

(91) Rana, S. V; Pal, R.; Vaiphei, K.; Sharma, S. K.; Ola, R. P. *Nutr. Res. Rev.* **2011**, *24*, 60–71.

(92) Borek, C. *J. Nutr.* **2001**, *131*, 1010S–1015.

(93) Qidwai, W.; Ashfaq, T. *Evidence-Based Complement. Altern. Med.* **2013**, 1–9.

(94) Ried, K.; Frank, O. R.; Stocks, N. P.; Fakler, P.; Sullivan, T. *BMC Cardiovasc. Disord.* **2008**, *8*, 13.

(95) Banerjee, S.; Maulik, S. *Nutr. J.* **2002**, *1*, 4.

(96) Kashfi, K.; Olson, K. R. *Biochem. Pharmacol.* **2013**, *85*, 689–703.

(97) Olas, B. *Chem. Biol. Interact.* **2014**, *217*, 49–56.

(98) Caron, F.; Kramer, J. R. *Anal. Chem.* **1989**, *61*, 114–118.

(99) Luthy, L.; Fritz, M.; Bachofen, R. *Appl. Environ. Microbiol.* **2000**, *66*, 712–717.

(100) Lippert, A. R. *J. Inorg. Biochem.* **2014**, *133*, 136–142.

(101) Chen, B.; Li, W.; Lv, C.; Zhao, M.; Jin, H.; Jin, H.; Du, J.; Zhang, L.; Tang, X. *Analyst* **2013**, *138*, 946–951.

(102) Qian, Y.; Zhang, L.; Ding, S.; Deng, X.; He, C.; Zheng, X. E.; Zhu, H.-L.; Zhao, J. *Chem. Sci.* **2012**, *3*, 2920–2923.

(103) Lippert, A. R.; New, E. J.; Chang, C. J. *J. Am. Chem. Soc.* **2011**, *133*, 10078–10080.

(104) Montoya, L. A.; Pluth, M. D. *Chem. Commun.* **2012**, *48*, 4767–4769.

(105) Lin, V. S.; Chang, C. J. *Curr. Opin. Chem. Biol.* **2012**, *16*, 595–601.

(106) Qian, Y.; Karpus, J.; Kabil, O.; Zhang, S.-Y.; Zhu, H.-L.; Banerjee, R.; Zhao, J.; He, C. *Nat. Commun.* **2011**, *2*, 495.

(107) Galardon, E.; Tomas, A.; Roussel, P.; Artaud, I. *Dalt. Trans.* **2009**, 9126–9130.

(108) Lynds, R.; Toner, R. *Wyoming State Geol. Surv.* **2014**.

(109) Coelho, A.; Castro, A. V; Dezotti, M.; Sant'Anna, G. L. *J. Hazard. Mater.* **2006**, *137*, 178–184.

(110) Environmental Protection Agency, U. S. *EPA Sets Tier 3 Motor Vehicle Emission and Fuel Standards*; **2014**; EPA–420–F–14–009.

CHAPTER 2

# AZIDOCOUMARIN COMPLEXES AS NOVEL PROBES FOR HYDROGEN SULFIDE *

## 2.1 Introduction

Hydrogen sulfide ($H_2S$) is a flammable gas, notorious for its distinct rotten egg-like odor and potent toxicity. While the majority of studies on the subject prior to the early 1980s focused on the negative health effects of $H_2S$ and those who work around it,[1–3] it has more recently come to light that this toxic small molecule or its downstream products are also important in cellular signaling and serve beneficial purposes in the inflammatory, cardiovascular, and nervous systems.[4–7] The majority endogenous hydrogen sulfide is dependent upon two human enzymes, cystathionine β–synthase (CBS) and cystathionine γ–lysase (CGL). Though it is clear that $H_2S$ plays critical roles in human physiology, the exact roles, pathways, and even concentrations of $H_2S$ are still unclear.[8–11]

One challenge that has dramatically slowed the advancement of this field is the lack of chemical probes with which to study $H_2S$ in biological systems. While many colorimetric[12–14] and fluorescent[9,15–18] probes have recently flooded the field, few have

* This chapter in an adaptation of both published work[37] and a manuscript recently accepted for publication.[43]

the sensitivity, [9,17] selectivity,[9] linear response,[19] or reaction speed[19] that is required for utility either as part of an enzyme assay or in high throughput settings. With the aim of meeting this challenge, we report the synthesis and characterization of two $H_2S$-responsive probes, AzMC and AzCC.

## 2.2 Materials and Methods

*2.2.1 General considerations*. All chemicals were purchased from commercial sources and used as received unless indicated otherwise. AzMC produced in our lab is now available from Sigma Aldrich (product number L511455) and Echelon Biosciences, Inc., Salt Lake City (product number D-0010). The truncated CBS enzyme (residues 1-413, CBS Δ1-413) was expressed in the Kraus lab as reported previously.[20] Tryptophan synthase was purchased from Sigma Aldrich. The $^{1}H$ and $^{13}C$ NMR data were collected on a Varian 400 MHz NMR spectrometer. Chemical shifts are reported in parts per million referenced to internal standard TMS = 0.00 ppm). Fluorescence and UV/Vis data were collected on a Molecular Devices Spectra Max M5.

*2.2.2 Synthesis of 7-azido-4-methylcoumarin, AzMC.* Precautions were taken to reduce the amount of light in the reaction. Foil was used to cover the flask, a septum was used to cover the mouth of the flask, and the hood lights were turned off. 7-amino-4-methylcoumarin (0.5 g, 1 equiv) was placed in water (11 mL) and allowed to stir at 0 °C in an ice/water bath. Concentrated sulfuric acid (3 mL) was added to the reaction in a dropwise fashion to maintain the temperature. Sodium nitrate (0.25 g, 1.2 equiv) was dissolved in water (3.4 mL), cooled to 0 °C, and added in a dropwise fashion to the reaction over 30 min. The mixture was then allowed to stir at 0 °C for 1 h. Meanwhile,

sodium azide (0.33 g, 1.76 equiv) was dissolved in water (2 mL) and cooled to 0 °C. After 1 h, the sodium azide solution was added to the reaction mixture in a dropwise fashion, and the formation of an off-white precipitate was immediately observed. The reaction mixture was then allowed to warm to room temperature overnight with stirring, and the solid was collected using a frit, washed with water (100 mL) and briefly air-dried. The resulting solid was then dissolved in chloroform, dried over anhydrous magnesium sulfate, and filtered, and the solvent was removed to yield a brown powder in 74.5% yield (0.44 g). $^{1}$H NMR (δ, $(CD_3)_2SO$) 2.41 (s, 3H), 6.33 (s, 1H), 7.15 (m, 2H), 7.77 (d, 1H). $^{13}$C NMR (δ, $(CD_3)_2SO$) 17.97, 106.64, 113.08, 115.39, 116.61, 126.82, 143.13, 152.76, 153.89, 159.39

*2.2.3 Synthesis of ethyl (3-hydroxyphenyl)carbamate,* ***1***. The commercial 3-aminophenol was recrystallized from hot water and again from hot toluene prior to use. Compounds **1**, **2**, and **ACC** were prepared using a modification of a previously published procedure.[21] An aliquot of 3-aminophenol (10 g, 1.0 equiv) in 35 mL ethyl acetate was refluxed for 30 min. Ethyl chloroformate (6.2 mL, 0.7 equiv) was added slowly over 30 min, resulting in the precipitation of a white solid. The reaction was refluxed 1 h longer. The precipitate was removed by vacuum filtration and washed with 2 x 3 mL ethyl acetate. The amber filtrate was concentrated under vacuum, and the product was recrystallized from hot isopropanol to give a white crystalline product in 41% yield (4.8 g). $^{1}$H NMR (δ, $(CD_3)_2SO$) 1.23 (t, 3H), 4.11 (q, 2H), 6.38 (d, 1H), 6.86 (d, 1H), 6.99-7.03 (m, 2H), 9.31 (s, 1H), 9.47 (s, 1H).

*2.2.4 Synthesis of 7-N-(carbethoxy)aminocoumarin-4-acetic acid,* ***2***. Ethyl (3-hydroxyphenyl)carbamate (**1**) (1.0 g, 1.0 equiv) was suspended in 22 mL of concentrated

$H_2SO_4$ at 0 °C. An aliquot of 1,3-acetone-dicarboxylic acid (0.9 g, 1.1 equiv) was added in three portions, and the mixture was allowed to warm to room temperature overnight. The resulting red-brown solution was poured over 33 g ice and stirred for 30 min. A cream-colored solid was collected by vacuum filtration, washed with 3 x 16 mL diethyl ether, and recrystallized from hot acetonitrile, yielding 1.5 g of product (89% yield). $^1$H NMR (δ, $(CD_3)_2SO$) 1.26 (t, 3H), 3.85 (s, 2H), 4.16 (q, 2H), 6.32 (s, 1H), 7.37 (d, 1H), 7.39-7.61 (m, 2H), 10.16 (s, 1H).

*2.2.5 Synthesis of 7-amino-4-carbamoylmethylcoumarin, ACC*. A 4 g aliquot (1 equiv) of 7-N-(Carbethoxy)aminocoumarin-4-acetic acid (**2**) was dissolved in 4 M sodium hydroxide (44 mL) and refluxed overnight. After cooling, the pH was adjusted to 2 using sulfuric acid, and the mixture was cooled to 4 °C. The resulting golden flaky precipitate was collected by vacuum filtration. Although the product was too wet to determine an accurate yield, it was carried on to the next step. $^1$H NMR (δ, $(CD_3)_2SO$) 3.73 (s, 2H), 5.98 (s, 1H), 6.17 (s, 2H), 6.42 (d, 1H), 6.56 (dd, 1H), 7.32 (d, 1H), 12.67 (s, 1H).

*2.2.6 Synthesis of 7-azido-4-carbamoylmethylcoumarin, AzCC*. 7-amino-4-carbamoylmethylcoumarin (**3**) (3.4 g, 1.0 equiv) was suspended in water (72 mL) at 0 °C followed by careful addition of 26 mL of concentrated $H_2SO_4$. A cold solution of sodium nitrite (1.3 g, 1.2 equiv) in 22 mL water was added in a dropwise fashion, and the solution was allowed to stir for 1 h at 0 °C. A cold solution of sodium azide (1.7 g, 1.7 equiv) in 13 mL water was added in a dropwise fashion, resulting in the immediate formation of a precipitate. The reaction was allowed to stir overnight, warming to room temperature. The brown-red solid was collected, washed with water, dissolved in

chloroform, and filtered again. The filtrate was dried over $MgSO_4$ and gravity filtered, and the solvent removed under vacuum to give 0.5 g of the final product (14% yield over two steps). $^1$H NMR (δ, $(CD_3)_2SO$) 3.91 (s, 2H), 6.44 (s, 1H), 7.15 (d, 1H), 7.18 (s, 1H), 7.71 (d, 1H).

*2.2.7 AzMC as a sensitive probe for hydrogen sulfide.* Sodium hydrogen sulfide was used as a convenient, readily measurable source of sulfide for these experiments. All fluorescence studies were carried out using 190 µL activity buffer (200 mM Tris HCl pH 8.0, 5 µM pyridoxal 5'-phosphate, 10 mM glutathione, 0.5 mg/mL BSA) and 10 µL DMSO, which was used to dissolved the 7-azido-4-methylcoumarin, unless otherwise specified. The probe was excited at 365 nm, and emission spectra at 450 nM were taken 1 h after mixing. A probe concentration of 10 µM was used while NaHS concentrations varied from 100 nM to 400 µM.

*2.2.8 AzMC as a selective probe for hydrogen sulfide.* A 10µM AzMC solution in buffer (5% DMSO was used to dissolve AzMC) was tested against a variety of thiols and reducing agents to determine selectivity for $H_2S$. AzMC was tested alone and in the presence of 100 µM NaHS, 10 mM- 30 mM cysteine, 10 mM- 30mM homocysteine, 1 mM PLP, 1 mM AdoMet, 100 µM- 1mM DTT, 100 µM- 1 mM TCEP, 10 mM glutathionine, and 10 mM BME.

*2.2.9 AzCC as a hydrogen sulfide probe*. As with AzMC, all fluorescence studies were carried out using 190 µL activity buffer (200 mM Tris HCl pH 8.0, 5 µM pyridoxal 5'-phosphate, 10 mM glutathione, 0.5 mg/mL BSA) and 10 µL DMSO, which was used to dissolve the AzCC, unless otherwise specified. The probe was excited at 365 nm, and emission spectra at 450 nM were taken 1 h after mixing. A probe concentration of 10 µM

was used while NaHS concentrations varied from 25 to 300 μM.

*2.2.10 Δ1-413 CBS enzyme activity assay using AzMC.* Truncated CBS activity studies were carried out in 190 μL activity buffer and 10 μL DMSO, which was used to dissolve the 7-azido-4-methylcoumarin. The probe concentration was 10 μM, the cysteine and homocysteine concentrations were each 2.5 mM, and 0.25 μg of CBS Δ1-413 (a final concentration of 27.8 nM) was used in a total volume of 200 μL. Immediately following the addition of substrates, the fluorescence of the mixture at 450 nm ($\lambda_{ex}$ = 365 nm) was monitored for 1 h. The plate reader was set up to automatically shake the plate for 30 s prior to the first data point and for 5 s between each data point (every min).

*2.2.11 Detection of CBS activity in crude cell extract using AzMC.* Three CBS mutations in bacterial cell extracts were obtained from the Kraus lab at UC-Denver. A 2.5 μg aliquot of protein was used in each experiment with 2.5 mM cysteine, 2.5 mM homocysteien, and 10 μM AzMC. (Indicated experiments contained 300 μM AdoMet.) The buffer consisted of 200 mM Tris, 0.5 mg/ml BSA, 5 μM PLP, 10 mM GSH, pH 8.0. The DMSO concentration was held constant at 5% total volume.

*2.2.12 Cystathionine beta-synthase (CBS) enzyme activity assay using AzMC or AzCC*. Both azidocoumarin probes were validated as assays to measure the activity of wild type human CBS. Several concentrations of CBS (0.25–5 μg) were tested against 10 μM probe, 2.5 mM cysteine, and 2.5 mM homocysteine in 190 μL buffer and 10 μL DMSO.

*2.2.13 Cystathionine gamma-lyase (CGL) enzyme activity assay using AzMC.* Experiments to monitor $H_2S$ production by CGL were carried out in 190 μL activity buffer with 10 μL DMSO used to dissolve the AzMC (10 μM). Cysteine and

homocysteine concentrations were each 2.5 mM, and 5 μg of CGL was used in a total volume of 200 μL. Immediately following the addition of substrates, the fluorescence of the mixture at 450 nm ($\lambda_{ex}$ = 365 nm) was monitored for 1 h. The plate reader was set up to automatically shake the plate for 30 s prior to the first data point and for 5 s between each data point (every min).

*2.2.14 Tryptophan synthase (TS) enzyme activity assay using AzMC.* Experiments to monitor $H_2S$ production by TS were carried out in 190 μL activity buffer with 10 μL DMSO used to dissolve AzMC. The probe concentration was 10 μM, the cysteine and homocysteine concentrations were each 10 mM and 20 μg of TS was used in a total volume of 200 μL. The probe was excited at 365 nm, and emission spectra at 450 nM were taken 1 h after mixing.

**2.3 7-Azido-4-Methylcoumarin (AzMC)**

Having experience with the coumarin scaffold,[22–26] we chose to base our work on the known fluorescent compound 7-amino-4-methylcoumain (AMC).[27–29] This fluorophore has been used extensively in enzyme assays and has been validated for use in such systems.[27] It has previously been established that aryl azides are readily reduced by hydrogen sulfide to form the amine.[16,18] We therefore hypothesized that azide derivatives of AMC, AzMC and AzCC, may have use as probes for $H_2S$.

*2.3.1 Design and synthesis of AzMC.* AzMC was synthesized using a modification of a previously reported procedure.[28] After reaction of AMC with sodium nitrite for 1 h in acid, the aryl azide was formed overnight using sodium azide. Unlike the amine and nitro derivatives, the azide is poorly soluble in acidic aqueous solution and readily precipitates

as it is formed. The product can then be collected by filtration and dried to give pure AzMC.

*2.3.2 Reactivity: sensitivity and selectivity.* With the goal of obtaining a facile fluorogenic assay for $H_2S$ that can be used as a chemical probe of $H_2S$ production, we had several specific properties in mind (Table 2.1). First, we required a probe with a broad linear range since serial dilutions could lead to increased errors and may not be practical in high throughput settings. We also required a probe sensitive enough to detect low levels of $H_2S$. While micromolar extracellular $H_2S$ levels have been reported,[10,30–32] it has been theorized that these concentrations could be grossly inflated. In fact, it has been proposed that $H_2S$ levels in tissues may be as low as 15 nM.[10,32,33] Lastly, we required a probe capable of detecting $H_2S$ in the presence of much higher concentrations of other biologically relevant thiols, reducing agents, and enzyme cofactors.

We began our investigation into AzMC by studying its reactivity with NaHS, a commonly used source of $H_2S$.[34] In contrast to the parent compound AMC, AzMC is minimally fluorescent. Upon reaction with NaHS, AzMC is reduced to form AMC with a concomitant increase in fluorescence (Figure 2.1). This signal is linear with respect to

Table 2.1 Desirable Qualities in a $H_2S$-Probe with Utility in Enzyme Assays

- Fluorescent
- Selective for $H_2S$ over other thiols and possible assay components
- Linear response to $H_2S$ over a broad range (at least 1–100 $\mu$M)
- Sensitive to $H_2S$: capable of detecting < 1 $\mu$M
- Fast acting, in that $H_2S$ can be detected in real time
- Compatible with a continuous assay format

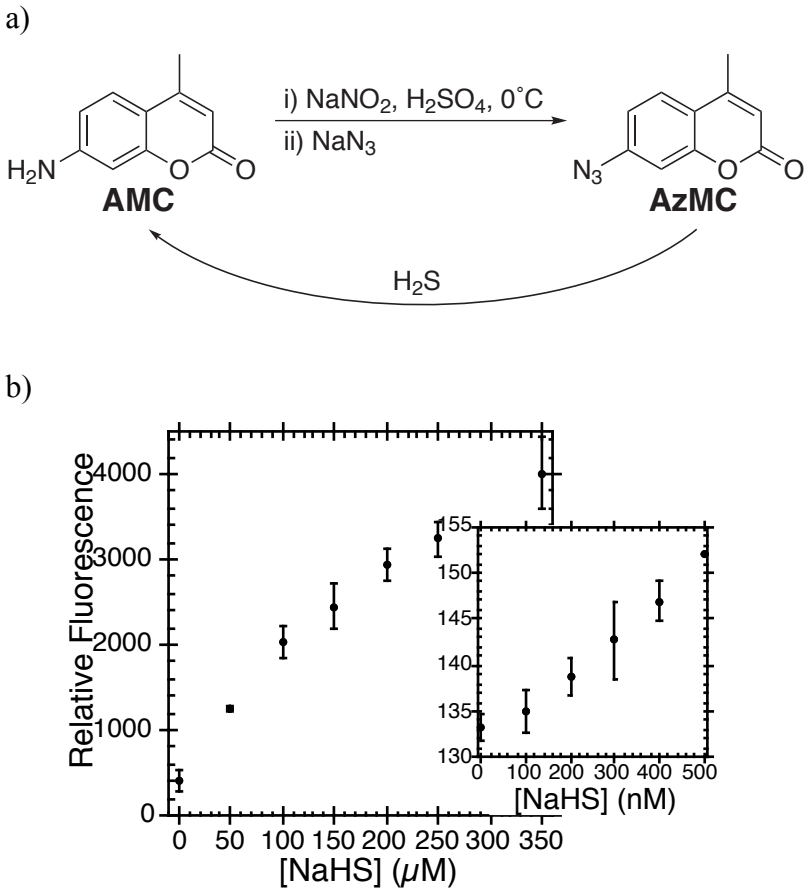



Figure 2.1 Synthesis and reactivity of AzMC. a) Synthetic scheme of AzMC. AzMC is reduced to AMC upon reaction with $H_2S$. b) Reduction of 10 μM AzMC with NaHS gives a linear signal from 100 nM to 100 μM NaHS as AMC is formed.

NaHS concentrations ranging from 200 nM to 100 μM NaHS and is an ideal dynamic range for enzyme assays and biological applications. These values are also to be expected as they are inline with those previously reported for other azide-based $H_2S$ probes,[16] including AzMC.[28]

We next explored the selectivity of AzMC for $H_2S$ over other thiols, reducing agents, and enzyme cofactors. As indicated in Figure 2.2, there was no response to 10 mM cysteine or homocysteine, 1 mM pyridoxial 5'-phosphate (PLP, the cofactor for CBS

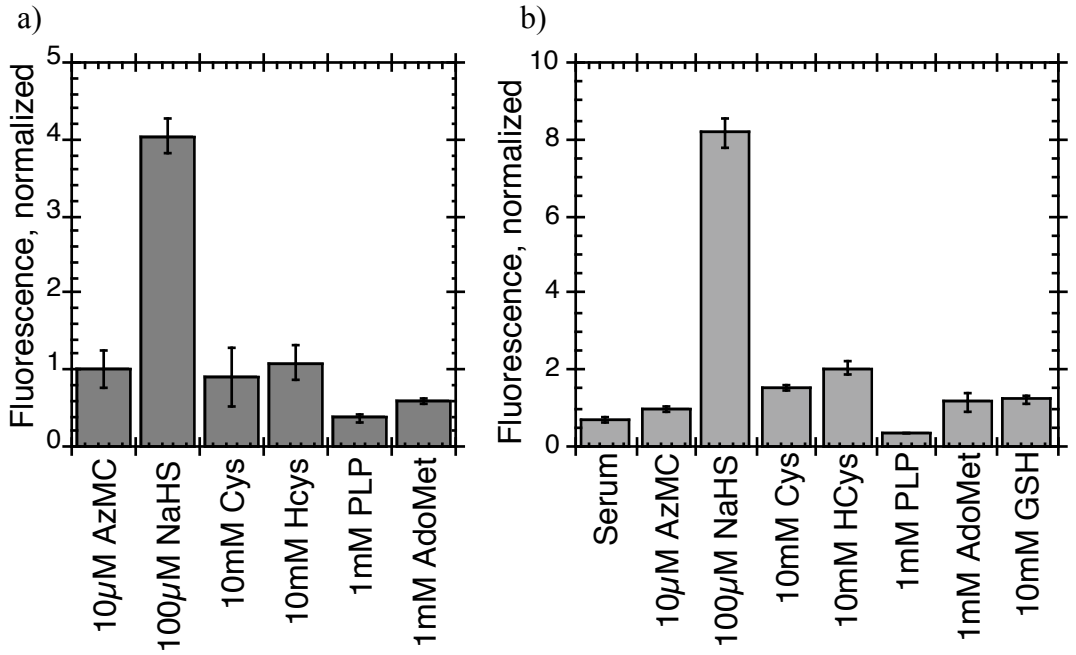



Figure 2.2 Selectivity of AzMC. a) AzMC is selective for $H_2S$ in activity buffer. b) AzMC is selective for $H_2S$ over other thiols in human serum.

and CGL) or 1 mM S-adenosyl-L-methionine (AdoMet), the allosteric activator of human CBS, though dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP) (common reducing agents with large negative reduction potentials) were capable of reducing AzMC. Two other reducing agents, glutathione and β-mercaptoethanol, (with smaller negative reduction potentials) did not interfere. Thus, while AzMC does not display absolute selectivity for $H_2S$, it does display enough selectivity to have utility in enzyme assays and biological samples provided care is taken to avoid DTT and TCEP.

Lastly, we explored the pH dependence of the AzMC reduction. While the background fluorescence of AzMC remained constant, over the range of pH tested (6.0–8.0), the reaction of AzMC with NaHS and AzMC with cysteine increased with

increasing pH. A maximum difference between the response for NaHS over cysteine was seen at pH 6.5; however, the maximum activity of CBS is reached at pH 8.0. Therefore, this pH was used for all experiments.

Because of the usefulness of AzMC for detecting hydrogen sulfide, AzMC synthesized in our lab is now commercially available through both Sigma Aldrich and Echelon, Inc. AzMC is doing quite well and is already profitable.

**2.4 7-Azido-4-Carbamoylmethylcoumarin (AzCC)**

*2.4.1 Design and synthesis.* Like AzMC, AzCC was also designed after AMC. However, by replacing the methyl at the 4-position with a carboxylic acid, we were able to instill AzCC with improved solubility in aqueous solutions. It has been shown that cystathionine γ-lysase[35] and I278T CBS[36], the most common missense CBS mutation, are sensitive to DMSO, and the sensitivity of CBS and CGL to high DMSO concentrations was, indeed, confirmed in our lab. It was, therefore, desirable to design a more soluble probe for use in such situations. Additionally, the carboxylic acid moiety could allow for conjugation with targeting agents such as peptides.

ACC was synthesized as described previously.[28] Briefly, 3-aminophenol was purified then reacted with ethyl chloroformate prior to a Pechmann condensation reaction to form the coumarin. After deprotection, the azide was formed from the amine as shown in Figure 2.3.[37]

*2.4.2 Reactivity of AzCC.* The reactivity of AzCC was studied using analogous approaches to those employed in the study of AzMC. It was determined that, like AzMC, NaHS is capable of reducing the azide in AzCC to form ACC. A similar linear response

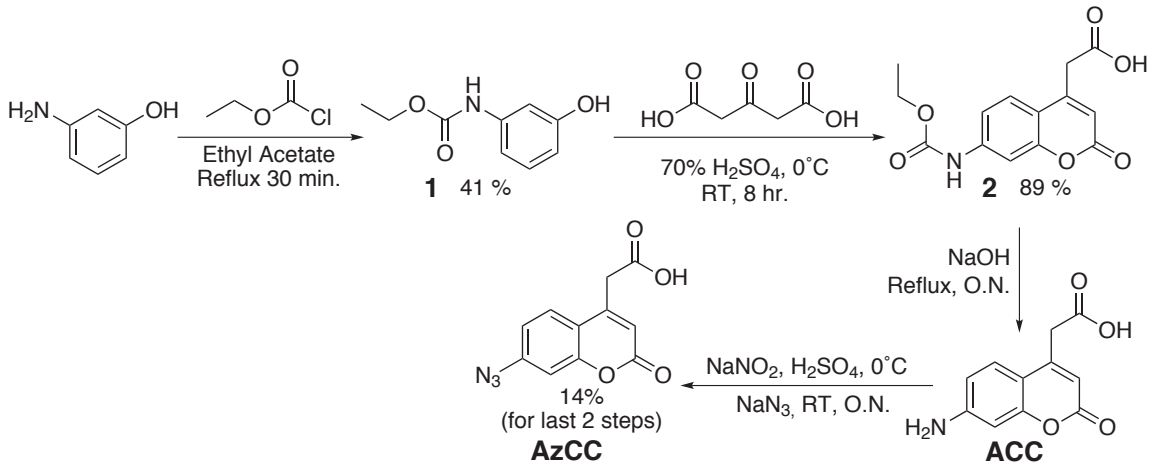



Figure 2.3 Syntheses of ACC and AzCC.

was seen, and, in fact, nearly identical fluorescent signals were produced (Figure 2.4).

## 2.5 Azidocoumarin Hydrogen Sulfide-Probes in Biological Conditions

The newly synthesized azidocoumarin probes should have many uses in biological studies. To validate these probes further, AzMC was tested under biological conditions (serum and crude bacterial extracts). Additionally, both probes were used in assays to study purified enzyme activity.

*2.5.1 Selectivity of AzMC in human serum.* In addition to studying the reactivity of AzMC with NaHS and other biologically relevant thiols in buffer, we were also interested in determining the selectivity of AzMC for $H_2S$ in human serum. We found the selectivity of AzMC to be consistent no matter the media (Figure 2.2).

*2.5.2 Detection of purified enzyme activity using azidocoumarin probes.* With two

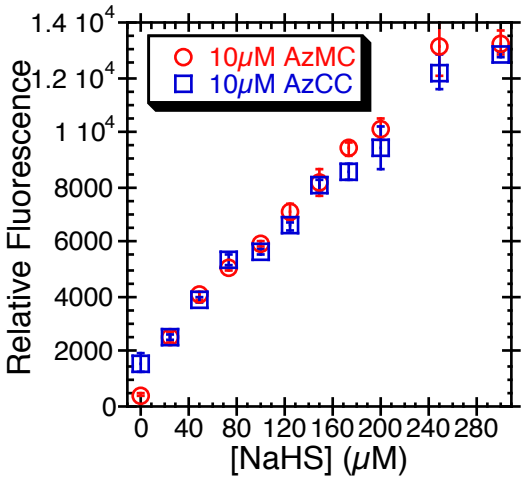



Figure 2.4 Response of 10 µM AzMC and 10 µM AzCC to NaHS. The reactivity of the two azidocoumarin probes are nearly identical.

$H_2S$-selective probes in hand, we set about designing an assay for $H_2S$-producing enzymes. By limiting the concentration of PLP (which quenches AMC) in the buffer and utilizing glutathione as the reducing agent, we were able to design an assay capable of detecting the release of $H_2S$ from enzymes in real time (Figure 2.5).

For simplicity, we began these studies by using a truncated version of human CBS, CBS Δ1-413, that does not contain the AdoMet-binding regulatory domain and has high constitutive activity in the absence of an activator.[20] As shown in Figure 2.5, significant signal over the background was recorded for CBS Δ1-413 activity using AzMC as the probe in the presence of cysteine and homocysteine. We then applied this assay to two other enzymes, the $H_2S$-producing enzyme CGL and tryptophan synthase (TS). TS is the prototypical type II fold PLP-dependent enzyme. In fact, TS shares the same highly conserved region as CBS, showing 28.5% sequence identity and 36% overall similarity.[38] While TS is known to catalyze the synthesis of tryptophan from serine and

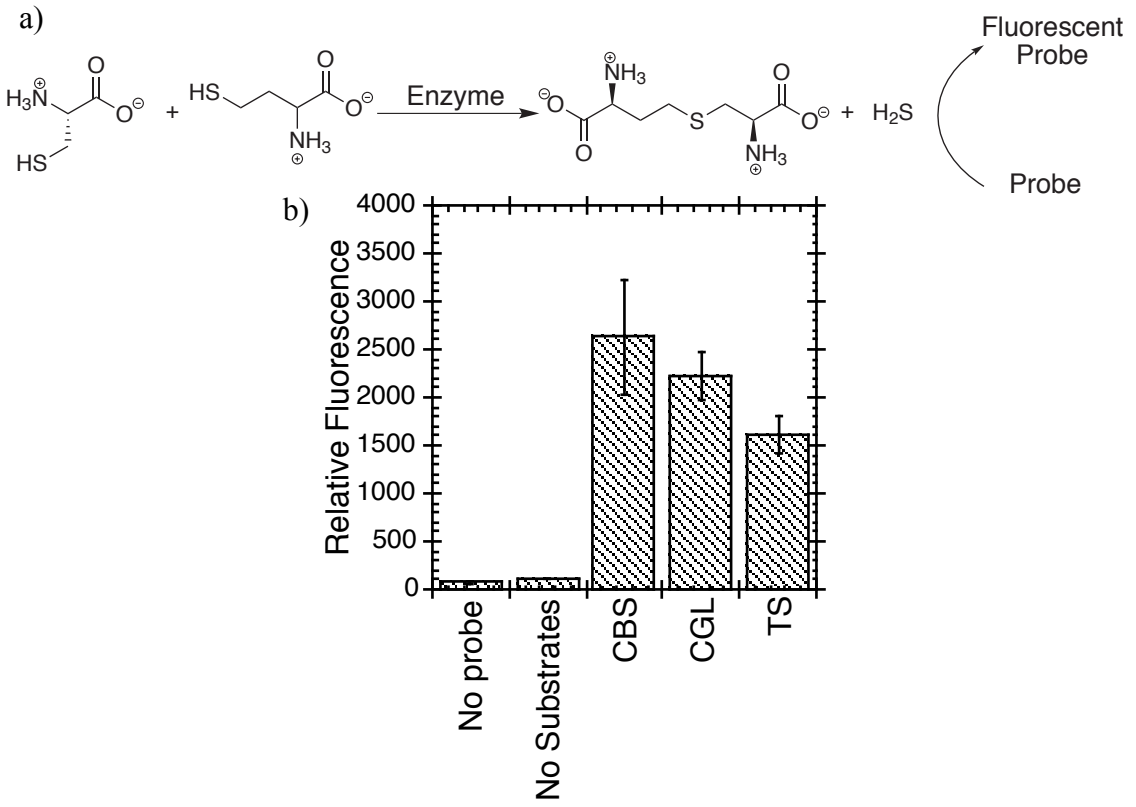



Figure 2.5 Azidocoumarins as tools to study enzyme activity. a) General enzyme assay scheme. b) AzMC (10 μM) as a tool for studying CBS Δ1-413 (5 μg), CGL (5 μg), and TS (20 μg).

indole in bacteria, yeasts, molds, and plants,[39] it had been shown that TS is capable of catalyzing a β-elimination reaction on cysteine to release $H_2S$.[40] Indeed, we were able to monitor the activity of both CGL and TS using AzMC in the presence of cysteine and homocysteine (Figure 2.5). Additionally, while the evolution of $H_2S$ can be observed from TS in the presence of cysteine alone, the addition of homocysteine promotes the reaction, likely by reacting with the α-aminoacrylate intermediate formed upon release of $H_2S$ to form cystathionine and thereby promoting catalytic turnover of the enzyme.

Lastly, we investigated the full length (WT) human CBS enzyme using AzCC.

While this enzyme is less active than the truncated mutant, activity was seen both with and without 300 µM AdoMet. Additionally, we saw a roughly two-fold increase in activity when AdoMet was present.

*2.5.3 Detecting CBS activity in crude bacterial cell extract.* In addition to purified enzyme, we are also interested in testing for low levels of endogenous CBS activity. Deactivating CBS mutations[41,42] can be a cause of homocystinuria (see Chapter 3). It is possible that our newly developed assay could be used to diagnose this genetic disorder in patient samples by determining the activity level of CBS.

After obtaining three samples containing distinct CBS mutants in crude bacterial extracts from the Kraus laboratory at the University of Colorado-Denver, we attempted to monitor CBS activity and its responsiveness to AdoMet using the AzMC assay (Figure 2.6). While the background signal was slightly higher in the crude extract than in buffer,

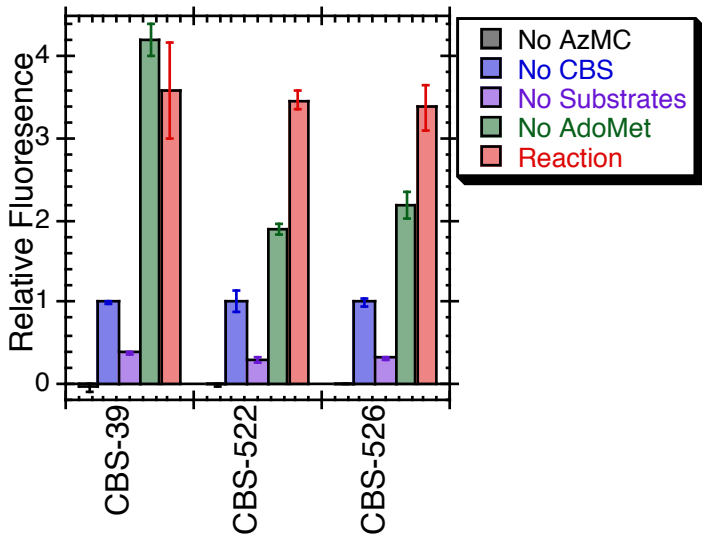



Figure 2.6 AzMC (10 µM) as a tool for studying CBS activity in crude bacterial cell extract (2.5 µg protein). CBS-522 and CBS-526 are AdoMet-responsive mutations, while CBS-39 is AdoMet-unresponsive—the addition of AdoMet does not appear to increase CBS activity.

we were still able to see enzyme activity over background under our assay conditions. Additionally, we detected increased activity levels in the presence of 300 μM AdoMet for the CBS-522 and CBS-526 mutations. Indeed these mutations were confirmed as AdoMet-responsive mutations using a radioactive serine-based assay, while CBS-39 was found to be AdoMet-unresponsive. These findings further validate AzMC and AzCC, not only as $H_2S$-selective probes, but additionally as tools with which to study enzyme activity.

## 2.6 Conclusion

We have designed and synthesized two hydrogen sulfide-selective probes based on the commonly used fluorophore AMC, AzMC and AzCC. These probes show selectivity for NaHS over other thiols (cysteine, homocysteine, glutationine, and β-mercaptoethanol), are sensitive to nanomolar concentrations of NaHS, and can be utilized over a range of pH conditions. Additionally, these probes can be used to provide a facile, sensitive, direct, and continuous assay for monitoring the activity of $H_2S$-producing enzymes, specifically CBS, CGL, and TS, both in buffer with purified enzyme and in crude cellular extracts. These probes may have utility in enzyme assay screens as we have shown that they are compatible under enzyme assay conditions. They may also have utility in industrial settings where the need for $H_2S$-detecting technology is great.

## 2.7 References

(1) Polhemus, D. J.; Lefer, D. J. *Circ. Res.* **2014**, *114*, 730–737.

(2) Ramazzini, B. *Am. J. Public Health* **1713**, *91*, 1380–1382.

(3) Knight, L. D.; Presnell, S. E. *Am. J. Forensic Med. Pathol.* **2005**, *26*, 181–185.

(4) Benavides, G. a; Squadrito, G. L.; Mills, R. W.; Patel, H. D.; Isbell, T. S.; Patel, R. P.; Darley-Usmar, V. M.; Doeller, J. E.; Kraus, D. W. *Proc. Natl. Acad. Sci.* **2007**, *104*, 17977–17982.

(5) Gallyas, F. *Br. J. Pharmacol.* **2012**, *166*, 2228–2230.

(6) Ishigami, M.; Hiraki, K.; Umemura, K.; Ogasawara, Y.; Ishii, K.; Kimura, H. *Antioxid. Redox Signal.* **2009**, *11*, 205–214.

(7) Li, L.; Rose, P.; Moore, P. K. *Annu. Rev. Pharmacol. Toxicol.* **2011**, *51*, 169–187.

(8) Whiteman, M.; Le Trionnaire, S.; Chopra, M.; Fox, B.; Whatmore, J. *Clin. Sci.* **2011**, *121*, 459–488.

(9) Montoya, L. A.; Pluth, M. D. *Chem. Commun.* **2012**, *48*, 4767–4769.

(10) Li, L.; Rose, P.; Moore, P. K. *Annu. Rev. Pharmacol. Toxicol.* **2011**, *51*, 169–187.

(11) Kolluru, G. K.; Shen, X.; Bir, S. C.; Kevil, C. G. *Nitric Oxide* **2013**, *35*, 5–20.

(12) Wang, S.-P.; Deng, W.-J.; Sun, D.; Yan, M.; Zheng, H.; Xu, J.-G. *Org. Biomol. Chem.* **2009**, *7*, 4017–4020.

(13) Montoya, L. A.; Pearce, T. F.; Hansen, R. J.; Zakharov, L. N.; Pluth, M. D. *J. Org. Chem.* **2013**, *78*, 6550–6557.

(14) Fischer, E. *Berichte Dtsch. Chem. Ges.* **1883**, *16*, 2234–2236.

(15) Galardon, E.; Tomas, A.; Roussel, P.; Artaud, I. *Dalt. Trans.* **2009**, 9126–9130.

(16) Lin, V. S.; Chang, C. J. *Curr. Opin. Chem. Biol.* **2012**, *16*, 595–601.

(17) Lippert, A. R.; New, E. J.; Chang, C. J. *J. Am. Chem. Soc.* **2011**, *133*, 10078–10080.

(18) Lippert, A. R. *J. Inorg. Biochem.* **2014**, *133*, 136–142.

(19) Qian, Y.; Karpus, J.; Kabil, O.; Zhang, S.-Y.; Zhu, H.-L.; Banerjee, R.; Zhao, J.; He, C. *Nat. Commun.* **2011**, *2*, 495.

(20) Frank, N.; Kent, J. O.; Meier, M.; Kraus, J. P. *Arch. Biochem. Biophys.* **2008**, *470*, 64–72.

(21) Zhu, Q.; Li, D. B.; Uttamchandani, M.; Yao, S. Q. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1033–1036.

(22) Stanford, S. M.; Krishnamurthy, D.; Kulkarni, R. A.; Karver, C. E.; Bruenger, E.; Walker, L. M.; Ma, C.-T.; Chung, T. D. Y.; Sergienko, E.; Bottini, N.; Barrios, A. M. *Methods* **2014**, *65*, 165–174.

(23) Hubbard, C. E. Chemical Probes of Protein Tyrosine Phosphatase Activity. Ph.D. Dissertation, University of Southern California, Los Angeles, CA, **2009**.

(24) Mitra, S.; Barrios, A. M. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 5142–5145.

(25) Mitra, S.; Barrios, A. M. *Anal. Biochem.* **2007**, *370*, 249–251.

(26) Mitra, S.; Barrios, A. M. *Chem. Bio. Chem.* **2008**, *9*, 1216–1219.

(27) Goddard, J.-P.; Reymond, J.-L. *Trends Biotechnol.* **2004**, *22*, 363–370.

(28) Chen, G.; Battaglia, E.; Senay, C.; Falany, C. N.; Radominska-Pandya, A. *Protein Sci.* **1999**, *8*, 2151–2157.

(29) Sivakumar, K.; Xie, F.; Cash, B. M.; Long, S.; Barnhill, H. N.; Wang, Q. *Org. Lett.* **2004**, *6*, 4603–4606.

(30) Zhang, M.; Shan, H.; Wang, T.; Liu, W.; Wang, Y.; Wang, L.; Zhang, L.; Chang, P.; Dong, W.; Chen, X.; Tao, L. *Neurochem. Res.* **2013**, *38*, 714–725.

(31) Kazuho, A.; Kimura, H. *J. Neurosci.* **1996**, *16*, 1066–1071.

(32) Kabil, O.; Banerjee, R. *J. Biol. Chem.* **2010**, *285*, 21903–21907.

(33) Chan, M. V; Wallace, J. L. *Am. J. Gastrointest. Liver Physiol.* **2013**, 467–473.

(34) Hughes, M. N.; Centelles, M. N.; Moore, K. P. *Free Radic. Biol. Med.* **2009**, *47*, 1346–1353.

(35) Zhang, L.; Liu, N.; Ma, X.; Jiang, L. *FEMS Yeast Res.* **2013**, *13*, 200–218.

(36) Singh, L. R.; Chen, X.; Kozich, V.; Kruger, W. D. *Mol. Genet. Metab.* **2007**, *91*, 335–342.

(37) Thorson, M. K.; Majtan, T.; Kraus, J. P.; Barrios, A. M. *Angew. Chemie Int. Ed.* **2013**, *52*, 4641–4644.

(38) Yanofsky, C.; Platt, T.; Crawford, I. P.; Nichols, B. P.; Christie, G. E.; Horowitz, H.; VanCleemput, M.; Wu, A. M. *Nucleic Acids Res.* **1981**, *9*, 6647–6668.

(39) Dunn, M. F.; Niks, D.; Ngo, H.; Barends, T. R. M.; Schlichting, I. *Trends Biochem. Sci.* **2008**, *33*, 254–264.

(40) Kayastha, A. M.; Miles, E. W. *Anal. Biochem.* **1991**, *193*, 200–203.

(41) Kraus, J. P. In *Sulfur and Sulfur Amino Acids*; Methods in Enzymology; Elsevier, 1987; Vol. 143, pp. 388–394.

(42) Kraus, J. P.; Janosík, M.; Kozich, V.; Mandell, R.; Shih, V.; Sperandeo, M. P.; Sebastio, G.; de Franchis, R.; Andria, G.; Kluijtmans, L. a; Blom, H.; Boers, G. H.; Gordon, R. B.; Kamoun, P.; Tsai, M. Y.; Kruger, W. D.; Koch, H. G.; Ohura, T.; Gaustadnes, M. *Hum. Mutat.* **1999**, *13*, 362–375.

(43) Thorson, M. K.; Van Wagoner, R. M.; Harper, M. K.; Ireland, C. M.; Majtan, T.; Kraus, J. P.; Barrios, A. M. **2014**, *submitted for publication.*

# CHAPTER 3

# IDENTIFICATION OF CYSTATHIONINE β–SYNTHASE INHIBITORS USING A HYDROGEN SULFIDE SELECTIVE PROBE [*]

## 3.1 Introduction

In Chapter 2 we discussed the design, synthesis, characterization, and investigation of two azidocoumarin complexes, AzMC and AzCC. These complexes have utility not only as sensors for low to moderate hydrogen sulfide ($H_2S$) levels but, additionally, as tools with which to monitor the activity of enzymes capable of producing $H_2S$: cystathionine β–synthase (CBS), cystathionine γ–lyase (CGL), and tryptophan synthase (TS).[1,2] CBS is of special interest because of the important roles it plays in human health. In addition to potential hydrogen sulfide production *in vivo,* it has been shown that CBS is the primary enzyme responsible for the regulation and metabolism of homocysteine, a toxic small molecule, in the brain and central nervous system.[3–7] Hyperhomocysteinemia, elevated serum homocysteine levels, has been associated with cardiovascular disease,[8–11] thrombosis,[12,13] neurodegenerative diseases,[5,6,14–16] and osteoporosis-like fractures.[17] It is not surprising, then, that similar effects are seen in

[*] This chapter is an adaptation of both published[2] work and a manuscript accepted for publication.[1]

patients with homocystinuria, a genetic condition resulting from deactivating CBS mutations. Characteristics of homocystinuria include an increased risk of vascular disease, mental retardation, lens dislocation, and skeletal abnormalities.[18,19] In addition to these homocysteine-related disorders, CBS has also been implicated in a number of other diseases ranging from Alzheimer's Disease[6] and Down's Syndrome[14] to hypertension[8] and preeclampsia.[20]

Despite the importance of CBS activity in cellular signaling and human health, remarkably few chemical probes are available with which to study its activity. Currently, there are only two widely recognized CBS inhibitors, aminooxyacetic acid (AOAA) and hydroxylamine (HA) (Figure 3.1).[21,22] Both have the ability to inhibit relevant CBS activity in the range of 1–10 mM;[23,24] however, both also target pyridoxial 5'-phosphate (PLP), the cofactor and reaction site of all three previously studied enzymes, namely CBS, CGL, and TS.[21,22] These compounds, therefore, are not selective for CBS over other PLP-dependent enzymes.[25] Additionally, only one biological activator of wild type human CBS is known, S-adenosyl-L-methionine, (AdoMet).[4,26] Not only could new activators potentially aid those suffering from homocystinuria and aid in the investigation of *in vivo* CBS activity, but such activators could help decipher the mechanism of CBS activation by providing some structure-activity relationship data.


**Aminooxyacetic acid** **Hydroxylamine**

Figure 3.1 Structures of known CBS inhibitors, aminooxyacetic acid (AOAA) and hydroxylamine (HA).

In an attempt to address these issues, we have used both AzMC and AzCC in screening efforts to identify novel CBS inhibitors and activators. We first used AzMC in the screen of a library of known bioactive and clinically used compounds against the highly active, truncated form of CBS, CBS Δ1-413, with the specific goal of discovering potent and selective inhibitors. We then applied AzCC in a smaller screen against the wild type human CBS, searching for both activators and inhibitors of CBS activity.

## 3.2 Materials and Methods

*3.2.1 General considerations.* All chemicals were purchased from commercial sources and used as received unless indicated otherwise. Fluorescence and UV/Vis data were collected using a Molecular Devices Spectra Max M5 plate reader. A subset of the Marine Invertebrate Compound Library (MICL-240) containing 240 compounds was provided by the Ireland lab via the University of Utah Drug Screening Resource Core Facility. The full length, wild type CBS enzyme,[27] truncated CBS enzyme (residues 1-413, CBS Δ1-413),[27] and CGL[28] were expressed and purified in the Kraus lab as reported previously. Tryptophan synthase was purchased from Sigma Aldrich. AzMC and AzCC were synthesized as reported in Chapter 2.[29,30] Unless otherwise indicated, enzyme activity buffer consists of 200 mM Tris HCl, 0.5 mg/mL BSA, 5 μM PLP, pH 8.0.

*3.2.2 Microsource Spectrum Library screen*. All studies were carried out in 185 μL activity buffer and 15 μL DMSO, which was used to dissolve both the 7-azido-4-methylcoumarin and the library compounds. The probe concentration was 10 μM, the cysteine and homocysteine concentrations were each 2.5 mM, the final concentration of each library compound was 50 μM, and 0.25 μg of CBS Δ1-413 was used in each well.

Immediately following the addition of substrates, the fluorescence of the mixture at 450 nm ($\lambda_{ex}$ = 365 nm) was monitored for 1 h. The plate reader was set up to automatically shake the plate for 30 s prior to the first data point and for 5 s between each data point (every min). Comparison of the screening results with a DMSO control was used to identify compounds that modified CBS activity in this screen.

*3.2.3 Microsource Spectrum Library dose-dependence hit validation*. Hit validation studies were carried out with 150 µL activity buffer and 50 µL DMSO, which was used to dissolve both the 7-azido-4-methylcoumarin and the compound. The probe concentration was 10 µM, cysteine and homocysteine concentrations were each 2.5 mM, and 0.25 µg CBS Δ1-413 was used to give a final concentration of 40 nM. The compounds were preincubated with the enzyme for 30 min. As before, immediately following the addition of AzMC and substrates, the fluorescence of the mixture at 450 nm ($\lambda_{ex}$ = 365 nm) was monitored for 1 h. The plate reader was set up to automatically shake the plate for 30 s prior to the first data point and for 5 s between each data point (every min). Relative rates were determined after background subtraction.

*3.2.4 Microsource Spectrum Library hit validation against NaHS.* Experiments designed to identify hits that react with NaHS rather than CBS were carried out in 180 µL activity buffer and 20 µL DMSO, which was used to dissolve both the 7-azido-4-methylcoumarin and the compound. For each inhibitor, the concentration of NaHS was held steady at 100 µM, the probe concentration was 10 µM, and the concentration of compound was varied from 0–100 µM. Each plate was incubated for 30 min prior to measuring fluorescence.

*3.2.5 Microsource Spectrum Library hit validation against AMC.* Experiments

designed to identify hits that react with AMC rather than CBS were carried out in 180 μL activity buffer and 20 μL DMSO, which was used to dissolve both the 7-azido-4-methylcoumarin and the compound. The concentration of AMC was fixed at 5 μM, and the compound concentration varied from 0–100 μM. The assays were incubated for 30 min before fluorescence was measured.

*3.2.6 Counterscreen of validated hits using CGL and TS.* To determine the selectivity of hits, identified inhibitors of CBS were counterscreened against CGL and TS. Experiments were either carried out using 150 μL activity buffer with 50 μL DMSO used to dissolve AzMC and the inhibitors for TS studies or 180 μL activity buffer with 20 μL DMSO for CGL. Inhibitor concentration was varied from 0 to 200 μM in the presence of 2.5 mM cysteine, 2.5 mM homocysteine, and either 3 μg CGL (375 nM) or 5 μg TS (714 nM).

*3.2.7 MICL-240 initial scree*n. The MICL-240 library of compounds was screened in duplicate using 10 μM AzMC, 0.25 μg WT CBS, 10 mM GSH, 2.5 mM Hcys, 2.5 mM Cys, and 2 μg of each compound in 100 μL of solution containing 4% DMSO, 200 mM Tris HCl, 0.5 mg/mL BSA, 5 μM PLP, pH 8.0.

*3.2.8 MICL-240 screen initial validation for dose-dependence.* Compounds capable of reducing wild type CBS activity to less than 50% in the initial MICL library screen were further validated at 4 μg compound using 10 μM AzMC, 2.5 mM Hcys, and 2.5 mM Cys, in 100 μL of solution containing 7% DMSO, 200 mM Tris HCl, 0.5 mg/mL BSA, 5 μM PLP, and 10 mM GSH, pH 8.0 both with and without 0.25 μg WT CBS. The no-enzyme rate was determined to be “background” and was subtracted for the CBS-induced rate. Additionally, both DMSO and 50 μM S-adenosylmethionine were used as

controls with all data standardized to background-subtracted DMSO controls.

*3.2.9 MICL-240 screen secondary validation for inhibition in the presence of AdoMet*. Compounds that demonstrated concentration-dependent inhibition of WT CBS based on the initial screen and validation were further tested at a set concentration of 200 μM. Each reaction had 100 μL total volume with 5% DMSO, 200 mM Tris HCl, 0.5 mg/mL BSA, 5 μM PLP, 10 mM GSH, pH 8.0. In addition, 10 μM AzCC, 2.5 mM Hcys, 2.5 mM Cys, 50 μM S-adenosylmethionine, and 200 μM compound were combined with or without 0.25 μg WT CBS. The no-enzyme rate was determined to be "background" and was subtracted for the CBS-induced rate. All data were standardized to background-subtracted DMSO controls. This experiment was designed to ensure that the compounds are capable of inhibiting enzyme activity in the presence of the endogenous CBS activator, S-adenosyl methionine (AdoMet).

*3.2.10 MICL-240 $IC_{50}$ determination.* Of the hits identified in the screening efforts described above, compounds that were available in both sufficient quantity and a >90% purity based on LC-MS were carried forward for dose-response studies. The $IC_{50}$ values of potential inhibitors of wild type CBS were determined using 0.25 μg WT CBS, 10 μM AzCC, 2.5 mM Cys, 2.5 mM Hcys, 300 μM s-adenosyl-methionine in 100 μL total volume (5% DMSO, 200mM Tris HCl, 0.5 mg/mL BSA, 5 μM PLP, 10 mM GSH, pH 8.0). As with the previous validations, the no-enzyme rate was determined to be "background" and was subtracted to obtain the CBS-induced rate. All data were standardized to background-subtracted DMSO controls.

*3.2.11 MICL-240 control experiments for false positives.* To ensure that the lead compounds are, in fact, inhibitors of CBS activity, a series of control experiments was

run. Any spurious reaction of the compounds with the enzyme, the azidocoumarin probe, or other components of the enzyme reaction mixture was controlled for by running enzyme-free controls and background subtracting. However, this protocol cannot rule out the possibility that the compounds react directly with the $H_2S$ produced by the enzyme, reducing the amount of $H_2S$ available to reduce the probe and ultimately reducing the fluorescent signal obtained. To investigate this possibility, we looked at the ability of the compounds to decrease the development of fluorescence of 10 μM AzCC in the presence of 100 μM NaHS (5% DMSO, 200mM Tris HCl, 0.5 mg/mL BSA, 5 μM PLP, 10 mM GSH, pH 8.0). Compounds MNP2-B7 and MNP2-A6 interfere slightly with the development of fluorescence at concentrations near their $IC_{50}$ values (~80 μM). However, this modest fluorescence decrease is not enough to account for the decrease in signal seen in the enzyme assays. Therefore we conclude that the compounds are indeed inhibiting enzyme activity.

### 3.3 Microsource Spectrum Collection Screen

We began our investigation into novel inhibitors of CBS Δ1-413 by screening 1900 compounds from the Microsource Spectrum Collection. This commercially available library contains compounds selected to provide a wide range of biological activities and structural diversity including approximately 50% clinically used drugs, 30% natural products from sources worldwide, and 20% compounds with known biological activities. AzMC was used for this initial screen as it was more readily synthesized and more thoroughly investigated. As mentioned previously, the truncated enzyme was used for this principle screen as it proved a simpler starting point than the

less active, AdoMet-responsive full length CBS. The library was screened in duplicate at 50 μM compound concentration, resulting in the identification of 70 compounds for further investigation. These results are depicted schematically in Figure 3.2.

*3.3.1 Initial hits.* Once initial inhibition properties were determined for all compounds at one concentration (50 μM), the effect of the lead inhibitors on the assay directly and on the enzyme at varying inhibitory concentrations was also investigated. In total, 12 compounds with significant inhibition at 150 μM (Figure 3.3) were validated. It is interesting that benserazide, a known inhibitor of the PLP-dependent enzyme DOPA decarboxylase,[31–33] came up as a top hit. While DOPA decarboxylase is not known to catalyze the production of $H_2S$, the fact that a known inhibitor of PLP-dependent enzymes came up as a hit in our screen gives us confidence in our assay.

*3.3.2 Inhibitor validation: false positives.* Though the one dozen lead compounds were validated in the absence of CBS and possessed concentration-dependent inhibition ($IC_{50}$), it is possible that several of the inhibitors interact with other components of our assay rather than directly with the enzyme. For example such "false positive" lead compounds could react directly with the hydrogen sulfide being detected, resulting in a lower readout despite their lack of interaction with CBS itself. Indeed, several of the top inhibitors contain electrophilic moieties that could display direct $H_2S$ reactivity. For example, piperine contains a classical Michael acceptor[34] and does react directly with NaHS during further testing. The compounds 1,4-naphthoquinone, 12a-hydroxy-5-deoxydehydromunduserone and alpha-mangostin also appear to interact directly with NaHS (Figure 3.4).

In addition to $H_2S$ reactivity, it is possible for lead compounds to quench the

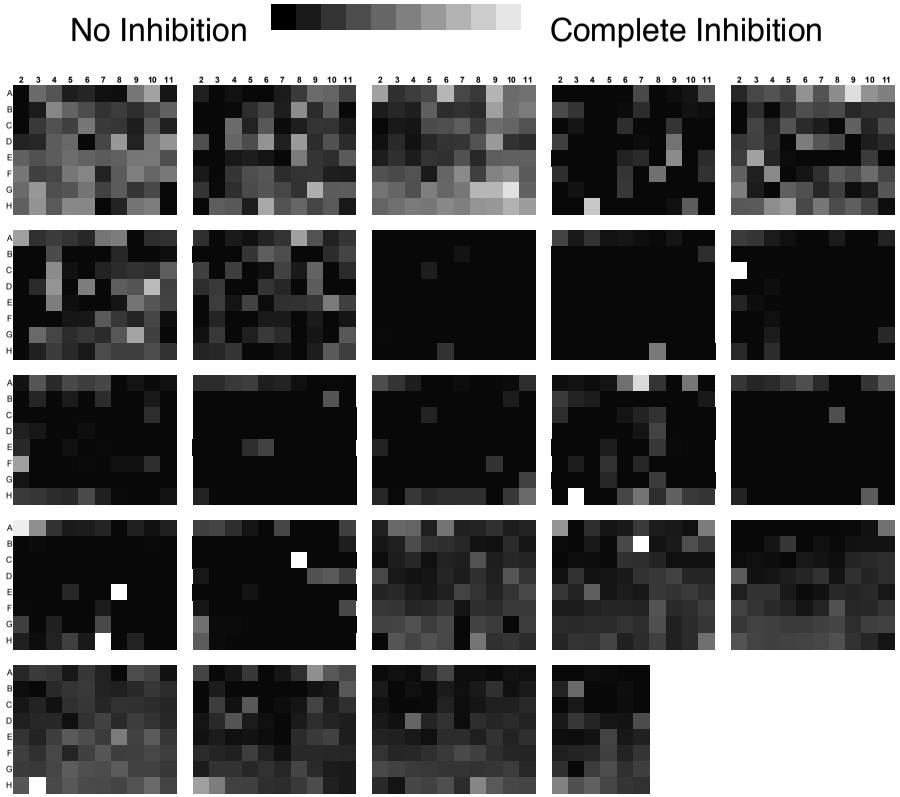



Figure 3.2 Heat map of Microsource Spectrum Collection screen against CBS Δ1-413. Black boxes indicate compound has no effect on CBS activity. White boxes indicate strong inhibitor effect.

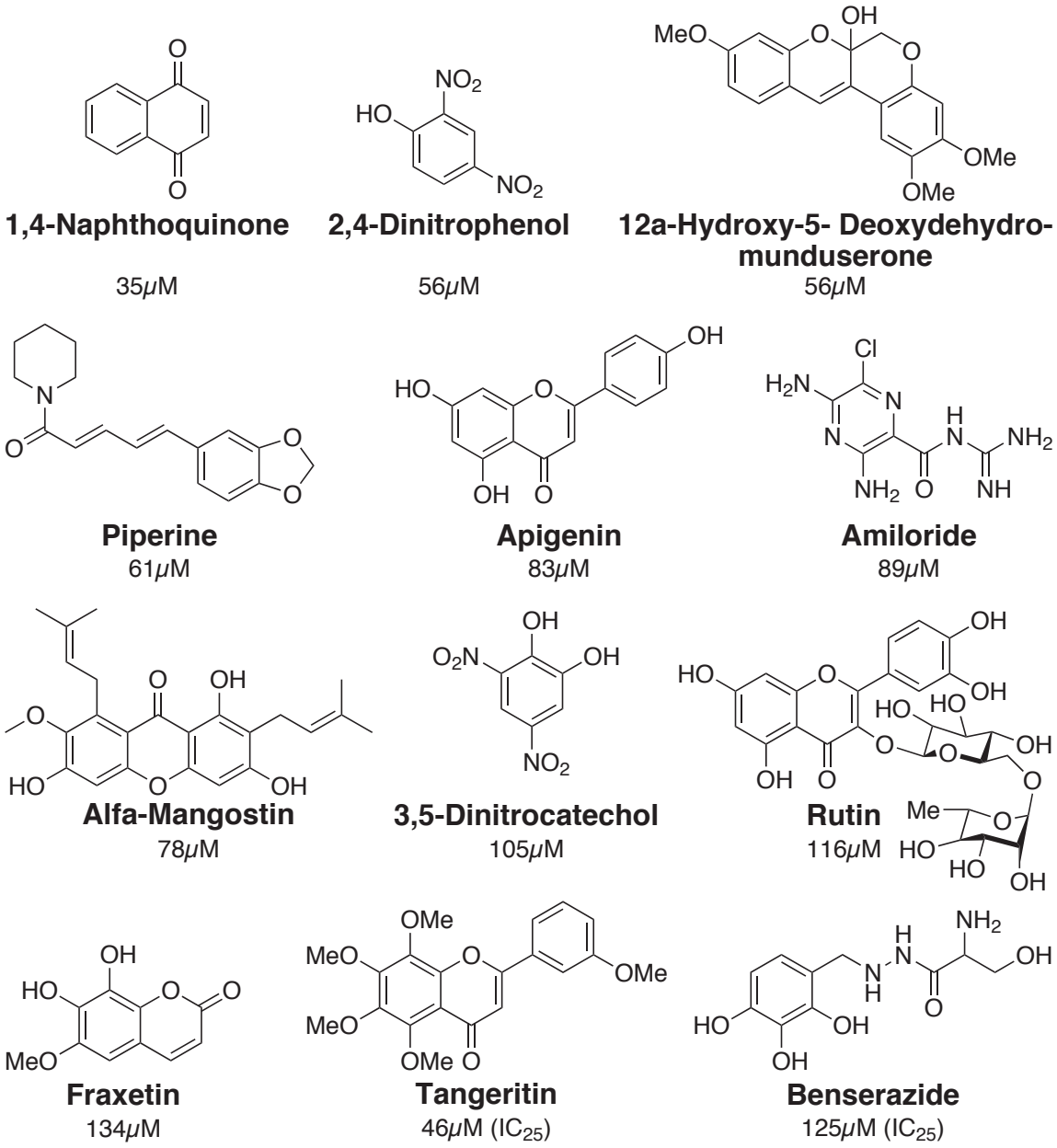



Figure 3.3 Lead Compounds from the initial validation of Microsource Spectrum Collection screen against 0.25 μg CBS using AzMC. Values represent $IC_{50}$ unless otherwise indicated.

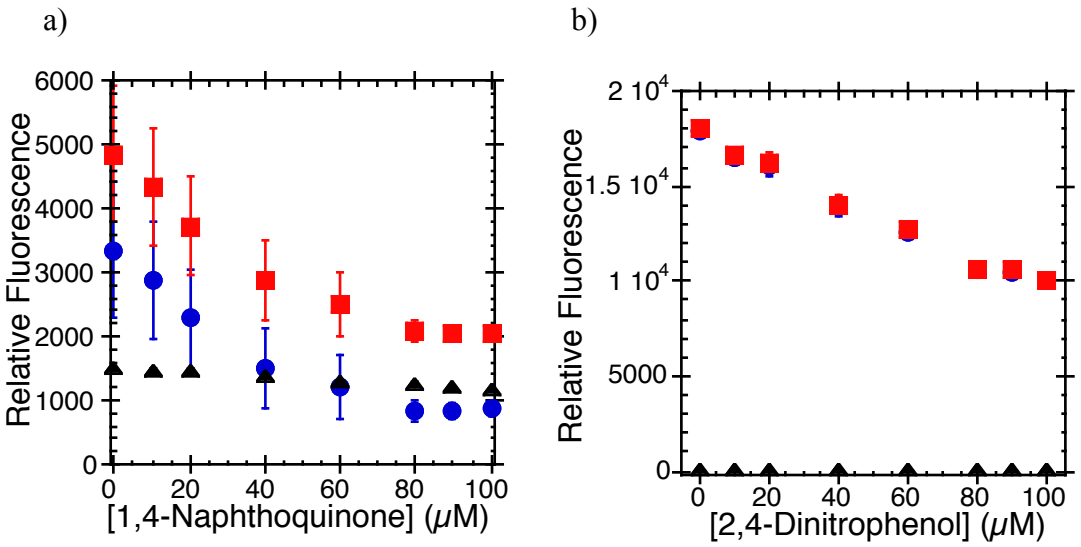



Figure 3.4 Examples of false positives discovered from the Microsource Spectrum library screen. a) 1,4-naphthoquinone in the presence of: 10 µM AzMC (black) alone, 10 µM AzMC and 100 µM (red), and corrected for background (blue). 1,4-naphthoquinone reacts with $H_2S$. b) 2-4-dinitrophenol alone (black), with 5 µM AMC (red), and corrected for background (blue). 2-4-dinitrophenol is an example of a compound that quenches the fluorescence of AMC.

fluorescence of AMC, resulting in a lower readout than the control while having no direct effect on the enzyme. Dinitrophenol and dinitrocatechol seem to quench the fluorescence of 5 µM AMC (Figure 3.4) and were therefore deemed "false positives."

*3.3.3 Inhibitor selectivity.* The selectivity of the previously validated CBS inhibitors for CBS over the closely related, PLP-dependent enzymes CGL and TS (Table 3.1) was determined using the same AzMC-based assay because these enzymes are also capable of catalyzing the production of $H_2S$ from cysteine and homocysteine as described in Chapters 1 and 2. While most of the compounds showed similar activity against all three enzymes, benserazide and fraxetin were significantly more potent against CGL than CBS. Tangeritin, on the other hand, was selective for CBS, displaying slightly less

Table 3.1: Selectivity of lead compounds showing $IC_{50}$ values in μM

| Compound | CBS | CGL | TS |
|---|---|---|---|
| 1,4-naphthoquinone | 35 | >>200 | 4 |
| α-mangostin | 78 | N/A | 38 |
| apigenin | 83 | 63 | 86 |
| amiloride | 89 | 71 | 74 |
| rutin | 116 | 73 | 90 |
| fraxetin | 134 | 73 | >>200 |
| tangertin | 46 ($IC_{75}$) | >>200 | 71 ($IC_{75}$) |
| Benserazide HCl | 125 ($IC_{75}$) | 102 | 66 ($IC_{75}$) |

potency towards TS than CBS and no inhibition towards CGL below 200 μM.

Interestingly, 1,4-naphthoquinone also showed no inhibition of CGL activity. This is counterintuitive with the assumption that 1,4-naphthoquinone is a false positive that reacts directly with $H_2S$ or NaHS. Taken together, our initial screening of a small chemical library yielded two compounds, 1,4-naphthoquinone and tangeritin, which specifically inhibit CBS over CGL.

### 3.4 The Marine Invertebrate Compound Library (MICL) Screen

Our screening of the Microsource Spectrum Collection against CBS Δ1-413 provided a nice starting point for the identification of novel CBS chemical probes and validated the azidocoumarin complexes as tools with which to study $H_2S$-producing enzymes in a high throughput setting. However, in our goal of discovering new *in vivo* modulators of CBS activity, we were interested in not only inhibitors, but activators as well. To this end, we turned our attention to the screen of wild type human CBS. This full length enzyme is less active than the truncated form, but contains the regulatory (AdoMet-binding) domain. It is therefore possible to screen this enzyme for compounds

that significantly increase activity in addition to those that inhibit activity.

Natural products isolated from marine invertebrates have been an exceptional source of chemical diversity and pharmaceutical lead compounds.[35–42] With the aim of streamlining the initial screening process while maximizing the chemical diversity that can be sampled, our collaborators in the Ireland lab created a protocol for fractionating marine invertebrate extracts.[43,44] They then selected a series of chemically diverse, well-characterized natural products and synthetic derivatives for use in screening. In the work reported in this section, we screened this subset of the "Marine Invertebrate Compound Library" consisting of 160 characterized marine natural products and 80 purified synthetic derivatives, termed "MICL-240," for compounds capable of activating or inhibiting CBS activity. From this library, we identified eight compounds with $IC_{50}$ values below 200 μM (range: 83–187 micromolar) and one activator.

*3.4.1 Initial results.* To begin our investigation, all 240 compounds were screened at a set concentration (200 μg / mL) against 0.5 μg wild type CBS using the soluble azidocoumarin, AzCC. As shown in Figure 3.5, several initial hits, both activators (green) and inhibitors (red), were identified. Compounds capable of reducing the activity of CBS by more than 50% were rescreened at 400 μg/ mL to confirm a dose-dependent response and validated in the presence and absence of enzyme to correct for any background fluorescence. The hits that demonstrated concentration-dependent modulation of enzyme activity were subjected to a series of secondary screens.

*3.4.2 Inhibitor validation.* In addition to the initial screens described in section 3.4.1, dose-response curves were measured for lead inhibitors in the presence of the endogenous CBS activator, S-adenosyl-L-methionine (AdoMet). We felt that inhibitors

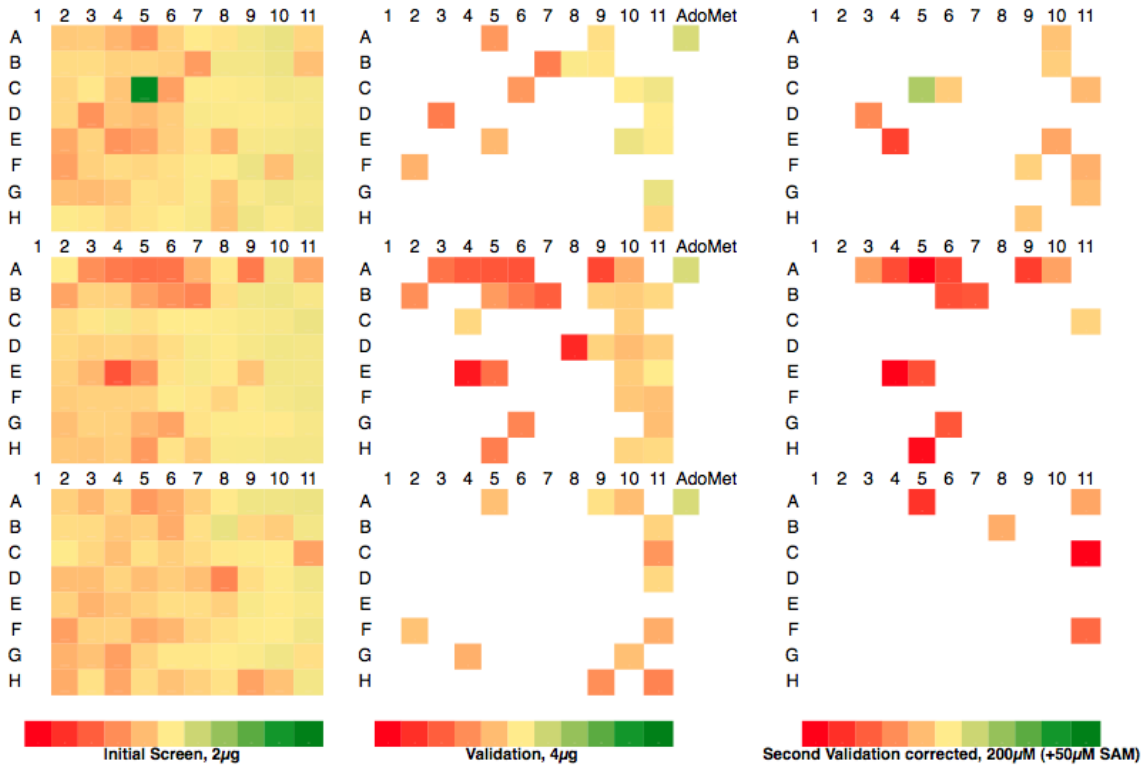



Figure 3.5 Heat map of MICL initial screen and further validations. Far left: initial screen at 2 µg compound. Middle panel: Inhibitors showing activity reduction greater than 50% were checked for concentration dependence using 4 µg compound. Data shown are not background corrected for ease of comparison with initial screen. Far right: Second validation. Compound inhibition was determined at 200 µM compound in the presence of 50 µM activator, AdoMet. Background (no enzyme control) of second validation was subtracted.

in the presence of low levels of AdoMet. These validated hits, with $IC_{50}$ values, are shown in Figure 3.6.

In an analogous manner to the Microsource Spectrum Collection screen, we also confirmed that our lead compounds were, in fact, directly interfering with the ability of CBS to metabolize cysteine and homocysteine rather than interfering with another part of the assay. None of the compounds shown in Figure 3.6 demonstrate interference with

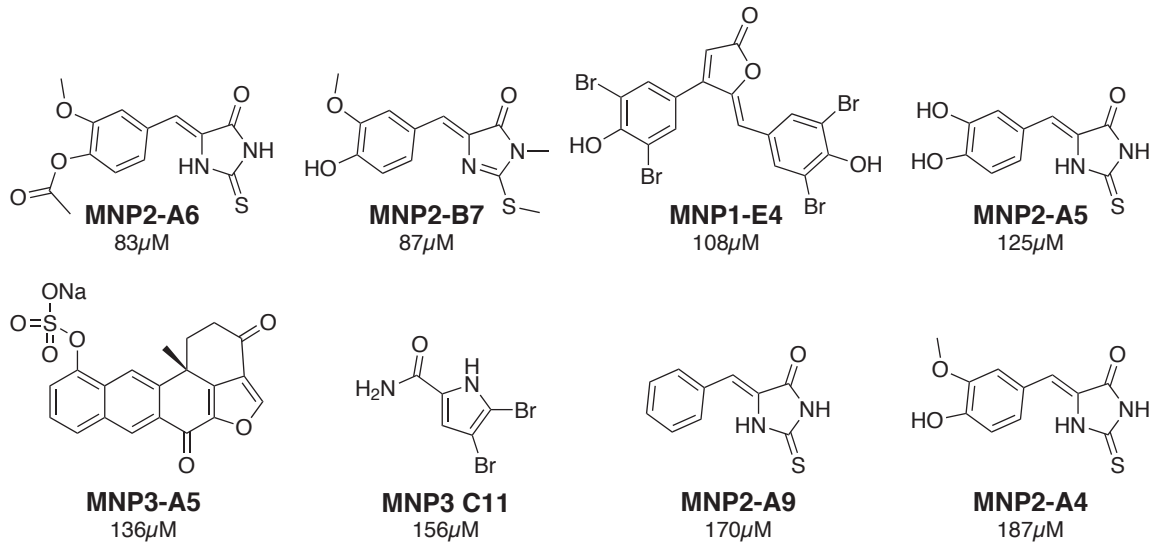



Figure 3.6 Validated inhibitors from MICL screen. Values are $IC_{50}$ (μM).

AzCC, ACC, or $H_2S$.

*3.4.3 Discussion of validated inhibitors*. It is interesting that the majority of lead inhibitory compounds (2-A6, 2-A9, 2-B7, and 3-C11) from our natural product screen are synthetic compounds derived from polyandrocarpamines A and B, 2-aminoimidazolone compounds isolated from the ascidian *Polyandrocarpa* sp.[45] The MICL-240 library contained 17 compounds from this structural class in plate 2 (wells A2-B8) yet only a subset displayed activity in our initial screen and only five displayed activity in both of our secondary screens (Figure 3.5).

An investigation into the structures of the validated leads compared to compounds that did not inhibit CBS activity (Figure 3.7) in the initial screen provides some information about the structure-activity relationship of these compounds. First, compounds containing only the thiohydantoin or aromatic moiety are not good inhibitors

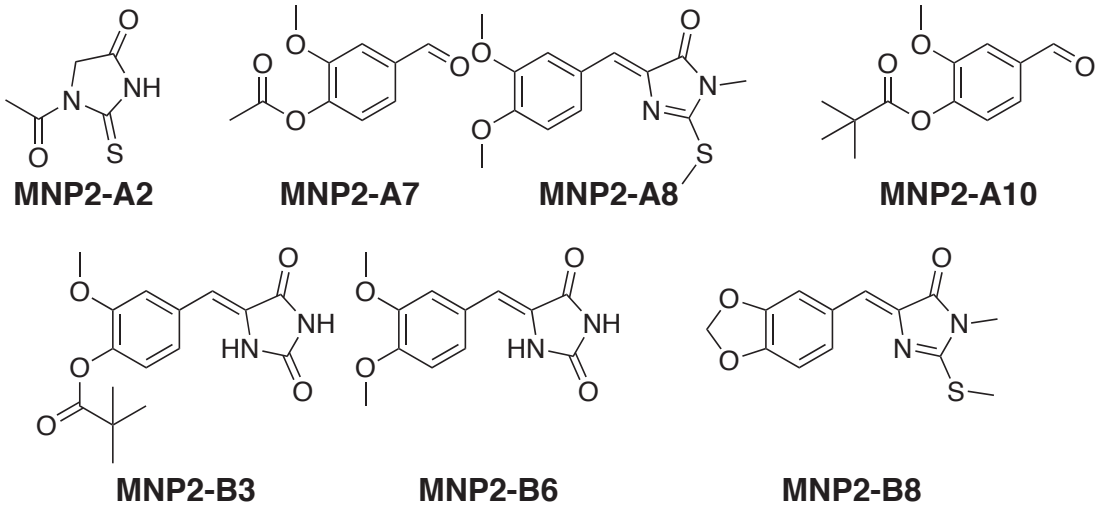
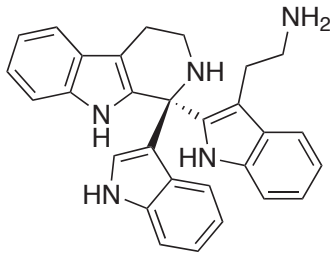



Figure 3.7 Structurally related compounds showing no inhibition against wild type CBS.

of CBS activity (compounds MNP2 A2, A7 and A10). Additionally, compounds MNP2-A8, MNP2-B6, and MNP2-B8, where both hydroxyl groups are etherified and the thiohydantoin either is methylated both at the 3-position and on the sulfur atom or replaced by a hydantoin moiety, do not show up as hits. Thiohydantoin methylation alone (compound MNP2-B7) does not prevent enzyme inhibition, while replacement of the thiohydantion moiety with a hydantoin moiety does (MNP2-B6, MNP2-B3).

*3.4.4 Potential CBS activator.* One of the most exciting results from the MICL screen with a broad range of potential implications was the identification of a novel CBS activator. A compound in well MNP1-C5 (Figure 3.8) increased wild type CBS activity by 329 ±19% in the initial screen. This increase was found to be concentration-dependent even after all background signals were removed and is equivalent or slightly greater than the activation of CBS by 500 μM AdoMet.[46,47] While the compound in MNP1-C5 was reportedly bengacarboline, a tetrahydro-β-carboline with a broad range of anticancer




**MNP1-C5**

Figure 3.8 Structure of bengacarboline, a potential activator of full length CBS

toxicities *in vitro*,[37,48,49] LC-MS analysis indicated that the compound had decomposed during storage. There was not enough sample available to identify the active component.

In an attempt to study structurally related compounds, we obtained a series of bengarcarboline derivates from the Pingaew lab.[38] Unfortunately, none of the synthetic derivatives were capable of activating CBS.

### 3.5 Conclusion

In Chapter 2 we explored the development of two $H_2S$-probes and their use in studying the activities of $H_2S$-producing enzymes. In this chapter, we have extrapolated these assays to include utility in high throughput screening. From the two screens described in this chapter we have identified a number of inhibitors more potent that those currently known. If it is in fact inhibiting CBS, 1,4-naphtoquinone would be the most potent inhibitor of CBS reported. While not quite as potent, the discovery of such compounds as alpha-mangostin, MNP2-A6, and MNP2-B7 is remarkable. These inhibitors show a marked improvement over the more traditional CBS inhibitors, AOAA and HA. Additionally, the discovery of a selective CBS inhibitor, tangertin, should open

the door to CBS-targeting experiments. In addition to the identification of novel CBS inhibitors, we also discovered a possible CBS activator. Though we were unsuccessful in identifying the structure of this compound, it gives us hope that small molecules other than AdoMet may be capable of enhancing CBS activity.

The aim of future work on this project will be to improve the potency and selectivity of CBS inhibitors and to identify activators of CBS activity. Larger, more diverse screens should aid in this endeavor. Ideal inhibitors will have an $IC_{50} < 10$ μM against CBS with a potency against CGL at least 10-fold higher. Acceptable activators will double CBS activity at 10 μM. Because the Pingaew library contained p-nitrobenzenesulfonyl derivatives of bengcarboline, it may be useful to screen a library of smaller tryptamines with similar structures to bengcarboline. It is possible that this added moiety prevented the screened compounds from binding and activating CBS. Additionally, serine or cysteine derivatives with a fluorinated α-carbon may also serve as potential CBS inhibitors, though most likely not selective inhibitors. These amino acid derivates would not be capable of a proton abstraction and would therefore trap the external aldimine. However, it may be more practical to synthesize derivatives of the 2-aminoimidazolone compounds based on our SAR findings.

### 3.6 References

(1) Thorson, M. K.; Van Wagoner, R. M.; Harper, M. K.; Ireland, C. M.; Majtan, T.; Kraus, J. P.; Barrios, A. M. **2014**. *submitted for publication.*

(2) Thorson, M. K.; Majtan, T.; Kraus, J. P.; Barrios, A. M. *Angew. Chemie Int. Ed.* **2013**, *52*, 4641–4644.

(3) Meier, M.; Oliveriusova, J.; Kraus, J. P.; Burkhard, P. *Biochim. Biophys. Acta* **2003**, *1647*, 206–213.

(4) Meier, M.; Janosik, M.; Kery, V.; Kraus, J. P.; Burkhard, P. *EMBO J.* **2001**, *20*, 3910–3916.

(5) Régnier, V.; Billard, J.-M.; Gupta, S.; Potier, B.; Woerner, S.; Paly, E.; Ledru, A.; David, S.; Luilier, S.; Bizot, J.-C.; Vacano, G.; Kraus, J. P.; Patterson, D.; Kruger, W. D.; Delabar, J. M.; London, J. *PLoS One* **2012**, *7*, e29056.

(6) Eto, K.; Asada, T.; Arima, K.; Makifuchi, T.; Kimura, H. *Biochem. Biophys. Res. Commun.* **2002**, *293*, 1485–1488.

(7) Kolluru, G. K.; Shen, X.; Bir, S. C.; Kevil, C. G. *Nitric Oxide* **2013**, *35*, 5–20.

(8) Nygård, O.; Vollset, S. E.; Refsum, H.; Brattström, L.; Ueland, P. M. *J. Intern. Med.* **1999**, *246*, 425–454.

(9) Blom, H. J.; Smulders, Y. *J. Inherit. Metab. Dis.* **2011**, *34*, 75–81.

(10) Polhemus, D. J.; Lefer, D. J. *Circ. Res.* **2014**, *114*, 730–737.

(11) Szabó, C. *Nat. Rev. Drug Discov.* **2007**, *6*, 917–935.

(12) Brattström, L.; Tengborn, L.; Lagerstedt, C.; Israelsson, B.; Hultberg, B. *Haemostasis* **1991**, *21*, 51–57.

(13) Ali, M.; Thomson, M.; Alnaqeeb, M. A.; Al-Hassan, J. M.; Khater, S. H.; Gomes, S. A. *Prostaglandins, Leukot. Essent. Fat. Acids* **1990**, *41*, 95–99.

(14) Ichinohe, A.; Kanaumi, T.; Takashima, S.; Enokido, Y.; Nagai, Y.; Kimura, H. *Biochem. Biophys. Res. Commun.* **2005**, *338*, 1547–1550.

(15) Shea, T. B.; Rogers, E. *N. Engl. J. Med.* **2002**, *346*, 2007–2015.

(16) Biselli, J. M.; Goloni-Bertollo, E. M.; Haddad, R.; Eberlin, M. N.; Pavarino-Bertelli, E. C. *Braz. J. Med. Biol. Res.* **2008**, *41*, 34–40.

(17) Van Meurs, J. B. J.; Dhonukshe-Rutten, R. A. M.; Pluijim, S. M. F.; van der Klift, M.; de Jonge, R.; Lindemans, J.; de Groot, Lisette, C. P. G. M.; Breteler, Monique, M. B.; Lips, P.; Pols, H. A. P.; Uitterlinden, A. G. *N. Engl. J. Med.* **2004**, *350*, 2033–2041.

(18) Kraus, J. P. In *Sulfur and Sulfur Amino Acids*; Methods in Enzymology; Elsevier, 1987; Vol. 143, pp. 388–394.

(19) Kraus, J. P.; Janosík, M.; Kozich, V.; Mandell, R.; Shih, V.; Sperandeo, M. P.; Sebastio, G.; de Franchis, R.; Andria, G.; Kluijtmans, L. a; Blom, H.; Boers, G. H.; Gordon, R. B.; Kamoun, P.; Tsai, M. Y.; Kruger, W. D.; Koch, H. G.; Ohura, T.; Gaustadnes, M. *Hum. Mutat.* **1999**, *13*, 362–375.

(20) Guzmán, M. a; Navarro, M. a; Carnicer, R.; Sarría, A. J.; Acín, S.; Arnal, C.; Muniesa, P.; Surra, J. C.; Arbonés-Mainar, J. M.; Maeda, N.; Osada, J. *Hum. Mol. Genet.* **2006**, *15*, 3168–3176.

(21) Li, L.; Moore, P. K. *Trends Pharmacol. Sci.* **2008**, *29*, 84–90.

(22) Li, L.; Whiteman, M.; Guan, Y. Y.; Neo, K. L.; Cheng, Y.; Lee, S. W.; Zhao, Y.; Baskar, R.; Tan, C.-H.; Moore, P. K. *Circulation* **2008**, *117*, 2351–2360.

(23) Yan, J.; Teng, F.; Chen, W.; Ji, Y.; Gu, Z. *Exp. Ther. Med.* **2012**, *4*, 832–838.

(24) Rashid, S.; Heer, J. K.; Garle, M. J.; Alexander, S. P. H.; Roberts, R. E. *Br. J. Pharmacol.* **2013**, *168*, 1902–1910.

(25) Whiteman, M.; Le Trionnaire, S.; Chopra, M.; Fox, B.; Whatmore, J. *Clin. Sci.* **2011**, *121*, 459–488.

(26) Banerjee, R.; Evande, R.; Kabil, Ö.; Ojha, S.; Taoka, S. *Biochim. Biophys. Acta* **2003**, *1647*, 30–35.

(27) Frank, N.; Kent, J. O.; Meier, M.; Kraus, J. P. *Arch. Biochem. Biophys.* **2008**, *470*, 64–72.

(28) Kraus, J. P.; Hasek, J.; Kozich, V.; Collard, R.; Venezia, S.; Janosíková, B.; Wang, J.; Stabler, S. P.; Allen, R. H.; Jakobs, C.; Finn, C. T.; Chien, Y.-H.; Hwu, W.-L.; Hegele, R. A.; Mudd, S. H. *Mol. Genet. Metab.* **2009**, *97*, 250–259.

(29) Chen, G.; Battaglia, E.; Senay, C.; Falany, C. N.; Radominska-Pandya, A. *Protein Sci.* **1999**, *8*, 2151–2157.

(30) Zhu, Q.; Li, D. B.; Uttamchandani, M.; Yao, S. Q. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1033–1036.

(31) Greenacre, J. K. K.; Coxon, A.; Petrie, A.; Reid, J. L. L. *Lancet* **1976**, *308*, 381–384.

(32) Rinne, U. K.; Birket-Smith, E.; Dupont, E.; Hansen, E.; Hyyppä, M.; Marttila, R.; Mikkelsen, B.; Pakkenberg, H.; Presthus, J. *J. Neurol.* **1975**, *211*, 1–9.

(33) Jonkers, N.; Sarre, S.; Ebinger, G.; Michotte, Y. *J. Neural Transm.* **2001**, *108*, 559–570.

(34) Avonto, C.; Taglialatela-Scafati, O.; Pollastro, F.; Minassi, A.; Di Marzo, V.; De Petrocellis, L.; Appendino, G. *Angew. Chemie Int. Ed.* **2011**, *50*, 467–471.

(35) Davidson, Bradley, S.; Molinski, T. F.; Barros, L. R.; Ireland, C. M. *J. Am. Chem. Soc.* **1991**, *113*, 4709–4710.

(36) Ford, P. W.; Narbut, M. R.; Belli, J.; Davidson, B. S. *J. Org. Chem.* **1994**, *59*, 5955–5960.

(37) Pouilhès, A.; Kouklovsky, C.; Langlois, Y.; Baltaze, J.-P.; Vispé, S.; Annereau, J.-P.; Barret, J.-M.; Kruczynski, A.; Bailly, C. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1212–1216.

(38) Pingaew, R.; Prachayasittikul, S.; Ruchirawat, S.; Prachayasittikul, V. *Arch. Pharm. Res.* **2012**, *35*, 949–954.

(39) Leal, M. C.; Madeira, C.; Brandao, C. A.; Puga, Joao, Calado, R. *Molecules* **2012**, *17*, 9842–9854.

(40) Senthilkumar, K.; Kim, S.-K. *Evidence-Based Complement. Altern. Med.* **2013**, 1–10.

(41) De Zoysa, M. *Adv. Food Nutr. Res.* **2012**, *65*, 153–169.

(42) Hussain, S.; Fareed, S.; Ansari, S.; Khan, S. *Indian J. Geo-Marine Sci.* **2012**, *4*, 27–39.

(43) Srivastava, V. C. *RSC Adv.* **2012**, *2*, 759–783.

(44) Soundararajan, S.; Badawi, M.; Kohlrust, C. M.; Hageman, J. H. *Anal. Biochem.* **1989**, *178*, 125–134.

(45) Davis, R. a; Aalbersberg, W.; Meo, S.; Rocha, R. M. Da; Ireland, C. M. *Tetrahedron* **2002**, *58*, 3263–3269.

(46) Janosík, M.; Kery, V.; Gaustadnes, M.; Maclean, K. N.; Kraus, J. P. *Biochemistry* **2001**, *40*, 10625–10633.

(47) Kery, V.; Bukovska, G.; Kraus, J. P. *J. Biol. Chem.* **1994**, *269*, 25283–25288.

(48) Foderaro, T. A.; Barrows, L. R.; Lassota, P.; Ireland, C. M. *J. Org. Chem.* **1997**, *62*, 6064–6065.

(49) Bedel, O.; Haudrechy, a.; Pouilhès, a.; Langlois, Y. *Pure Appl. Chem.* **2005**, *77*, 1139–1152.

# CHAPTER 4

# BENZOTHIEPINS AS HYDROGEN SULFIDE-DONATING COMPOUNDS

## 4.1 Introduction

As described in pervious chapters, hydrogen sulfide ($H_2S$) appears to be a salubrious gas at low micromolar concentrations, serving as a signaling molecule in numerous human organs, specifically the brain[1–6], cardiovascular system[7–12], and gastrointestinal tract.[13–15] Given their importance to human health, it is not surprising that reactive sulfur species such as $H_2S$ and the transsulfuration pathway have become potential targets for new therapeutics. One can imagine two drug classes that might attenuate $H_2S$ levels *in vivo*: those that moderate the activity of $H_2S$-producing enzymes and those that release $H_2S$ either directly or indirectly. The first type of potential therapeutic was discussed in Chapter 3. These compounds can activate or, more often, inhibit CBS and CGL activity. While such compounds were designed primarily for use as chemical probes for endogenous reactive sulfur species such as $H_2S$, it is not unreasonable to propose that potent, selective inhibitors might one day see use as therapeutics for CBS-upregulated diseases. The second class of potential $H_2S$-targeted therapeutics, those that directly increase cellular concentrations of reactive sulfur species, will be discussed and investigated in this chapter.

*4.1.1 Hydrogen sulfide-releasing agents.* Sulfide salts, such as NaSH, $Na_2S$, and CaS, can release hydrogen sulfide and are frequently used as sources of $H_2S$ in biological studies. While these compounds are simple, readily available, and soluble in aqueous solutions, they are not ideal for use as therapeutics. Sulfide salts produce a quick and often intense burst of sulfide rather than the slow, controlled released desired in a therapeutic.[16,17] Additionally, some studies report no change in plasma $H_2S$ levels upon injection of NaHS,[10] possibly due to the immediate oxidation of sulfide into reduction labile pools.[1] Ideally, a potential drug would have a consistent $H_2S$-release rate with a small concentration of $H_2S$ given off over a long time period. To accomplish this, attention has turned to hydrogen sulfide-donating compounds, that is, synthetic or naturally occurring complexes that raise sulfide levels through some reaction or metabolic pathway within the cell.[18] There are two types of such donating compounds: those that occur naturally, often in the food we eat, and those that are designed and synthesized by chemists.

*4.1.2 Naturally occurring hydrogen sulfide-donating compounds*. Many of the foods we eat, such as garlic, wasabi, horseradish, and broccoli, may contain compounds that produce $H_2S$ when metabolized. The consumption of garlic, for example, has long been known to be beneficial to human health, especially in the $H_2S$-sensitive cardiovascular and anti-inflammatory systems. In fact, these physiological effects may be the result of organosulfur-rich garlic being metabolized to produce $H_2S$. Garlic contains diallyl thiosulfinate, which is broken down to form the polysulfides diallyl disulfide (DADS) and diallyl trisulfide (DATS) (Figure 4.1).[11,17,19,20] It has been shown that these polysulfides undergo a reaction with reduced thiols such as glutathione to produce $H_2S$.[11]

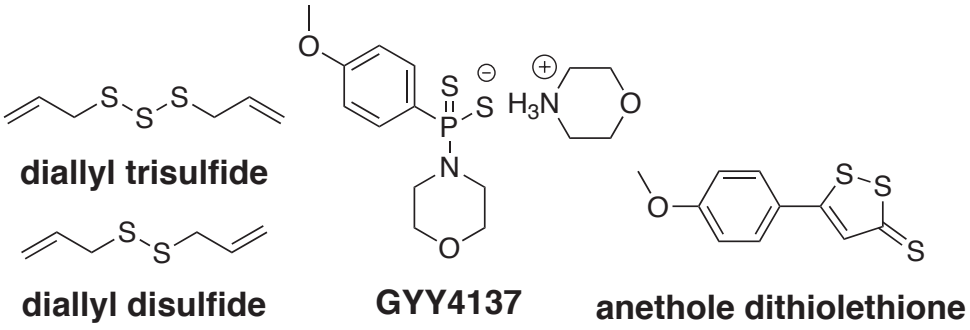



Figure 4.1 Structure of known $H_2S$-donating and -releasing compounds.

Further validating this idea is the finding that injection of DADS into mice increases the concentration of exhaled $H_2S$.[21] More recently, DATS has been used in a clinical trial of 5000 patients, giving promising results. The trial found that those taking a DATS supplement (200 mg/day) had 22% less instance of cancer over a 5-year period and a 47% less instance of gastric cancer.[19,22] This result could be due to reduction-induced hydrogen sulfide release as initially reported or through some other reactive sulfide intermediate such as persulfides.[23] In either case, this result underscores the importance of reactive sulfide complexes to human health and the importance of developing new $H_2S$-donating therapeutics.

*4.1.3 Synthetic hydrogen sulfide-donating compounds*. In addition to naturally occurring species, new $H_2S$-donating agents have also been designed and synthesized. One of the first complexes developed was GYY4137 (Figure 4.1), a derivative of Lawson's reagent. While GYY4137 does produce the desired slow release of $H_2S$, it is not overly efficient, giving off under 10% $H_2S$ in 90 minutes at pH 3.0 and less than 1% at physiological pH.

One of the most promising $H_2S$-donating compounds to date is anethole

dithiolethione (ADT). ADT contains an aromatic $H_2S$-producing motif that is readily bound to other drugs through an ether linkage. The ADT-derivative of many therapeutics have been synthesized and tested including, L-DOPA, sildenafil, and numerous NSAID pain relievers.[17] The negative effects of NSAIDs on the gastrointestinal tract were mitigated by the addition of this $H_2S$-donating moiety,[13] most likely because $H_2S$ aids in mucosal defense and repair.

Given the therapeutic potential, we set out to discover new $H_2S$-donating agents. These compounds should be trigged by some biological process or condition to produce $H_2S$ under cellular conditions rather than in buffer alone. Additionally, these complexes need to be nontoxic. Such compounds should prove useful in studying the effects of $H_2S$ and may have therapeutic potential in the treatment of a number of $H_2S$-related disorders.

## 4.2 Materials and Methods

*4.2.1 General considerations.* All chemicals were purchased from commercial sources and used as received unless indicated otherwise. The $^1$H NMR data were collected on a Varian 400 MHz NMR spectrometer. Chemical shifts are reported in parts per million referenced to internal standard $(CH_3)_4Si$ = 0.00 ppm. Fluorescence and UV/Vis data were collected using a Molecular Devices Spectra Max M5 plate reader.

*4.2.2 Initial screen of thiuram disulfide library using AzMC*. A library of thiuram disulfide was screened at 100 μM in 95% activity buffer (50 mM BisTris, 100 mM NaCl, pH 6.5) and 5% DMSO using 10 μM AzMC. Reactions took place in buffer alone, or with a reducing agent present (10 mM glutathione, 250 μM cysteine, or both). After 1 h incubation at room temperature, the fluorescence was measured according to the AzMC

procedure established in Chapter 2.

*4.2.3 Methylene blue assay.* The methylene blue assay was conducted as indicated in Chapter 5. Reaction mixtures were prepared in a 500 µL well plate in triplicate. A 3 µL aliquot of thiuram disulfide (10 mM stock) in DMSO was diluted in 297 µL buffer (50 mM BisTris, 100 mM NaCl, 0.01% Brij 35, pH 6.5) either with or without 2 mM glutathione. The plate was sealed with foil tape and left at room temperature for 24 h. For the methylene blue assay, a 70 µL aliquot from each well was reacted with 60 µL 10% w/v trichloroacetic acid, 30 µL 1% w/v zinc acetate (aq), and 20 µL 30 mM ferric chloride (in 1.2 M HCl). Addition of 20 µL 20 mM *N,N*-dimethyl-*p*-phenylenediamine sulfate (in 7.2 M HCl) yielded a blue color, which was detected after 10 min at 670 nm.

*4.2.4 Reducing agent dependence.* The reactivity of **4C** (100 µM) was studied in presence of 2 mM reductant (glutathione, cysteine, BME, DTT, and TCEP) and alone (control). A background control was run for each, substituting DMSO for **4C**. The data discussed are background corrected for the DMSO control. Each reaction, performed in triplicate, contained 99 µL buffer (50 mM BisTris, 100 mM NaCl, 0.01% Brij 35, pH 6.5) and 1 µL DMSO and was incubated for 1 h prior to testing. As described in section 5.2.3, a 70 µL aliquot of each was used in the methylene blue assay.

*4.2.5 pH dependent production of $H_2S$ from **4C**.* Using the same stock solution, six pH buffers were made (6.5–9.0) containing 50 mM Bis Tris Propane, 100 mM NaCl, and 0.01% Brij 35. In 100 µL final volume were placed 1 µL DMSO (used to dissolve **4C**, 100 µM) and 2 mM cysteine. (The remaining volume was made up from buffer.) As in section 4.2.4, a DMSO control was used to calculate background. After 1 h reaction time, each reaction was tested using the methylene blue assay.

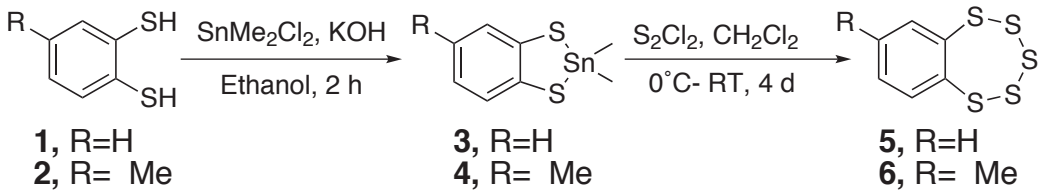
*4.2.6 Sulfolane controls.*The reactivity of **4C** (100 μM) was compared to that of DMSO and sulfolane (100 μM) in the presence and absence of 2 mM cysteine. The reactions took place in 99 μL buffer (50 mM Tris HCl, 100 mM NaCl, 0.1% Brij 35, pH 8.0) and 1 μL DMSO and was incubated 1 h prior to testing with methylene blue. An absorbance reading was taken 10 min after mixing of methylene blue assay.

*4.2.7 Comparison of $H_2S$-donating compounds with reducing agents.* GYY4137, **4C**, DADS, and DMSO (control) (100 μM) were reacted with 5 mM reducing agent (TCEP, glutathione, cysteine, BME, DTT, and buffer [control]) in 1 μL DMSO and 97 μM buffer (50 mM Tris HCl, 100 mM NaCl, 0.01% Brij 35, pH 8.0). After 2 h reaction, the methylene blue assay was used to determine $H_2S$ concentration.

*4.2.8 Kinetic comparison of $H_2S$-donating compounds.* In 193 μL buffer (50 mM Tris HCl, 100 mM NaCl, 0.01% Brij 35, pH 8.0) and 7 μL DMSO were dissolved 100 μM **4C**, DADS, or GYY4137, 2 mM cysteine (or buffer for controls), and 10 μM AzMC. The fluorescence of the plate was read at 450 nm (exciting at 365 nm) immediately following the addition of the $H_2S$-donating compounds.

*4.2.9 Purification of 4C.* An 11.73 mg sample of **4C** was dissolved in DMSO and $CHCl_3$ and dried onto silica before the solvent was removed. A silica column was run, eluting with 2% MeOH in $CHCl_3$. Two products were collected, each a yellow oil. The masses of each was determined using GC-MS, and the activity of each in the presence of glutathione was determined.

*4.2.10 Synthesis of dimethyltin compounds, **3** and **4**.* Compounds **3** and **4** were synthesized according to previously published methods (Figure 4.2)[24,25] Briefly, benzenedithiol (0.16 g, 1 equiv) was dissolved in ethanol (30 mL), and a 0.2 M KOH




**1,** R=H
**2,** R= Me
**3,** R=H
**4,** R= Me
**5,** R=H
**6,** R= Me

Figure 4.2 Synthetic scheme for synthesis of benzopentathiepins **5** and **6**.

solution in ethanol (14 mL) was added and allowed to stir for 30 min. An aqueous solution of dimethyltin chloride (0.56 g, 1.5 equiv) was added and allowed to stir for 3 h. The solvent was removed before the resulting solid was dissolved in $CH_2Cl_2$ (40 mL) and washed with water (2 x 20 mL) and brine (1 x 13 mL). The organic layer was collected, dried over anhydrous $MgSO_4$, filtered, and the solvent removed yielding **3** as a while solid in 33% yield. $^1$H NMR (δ, $CDCl_3$) 1.008 (s, 6H), 6.885 (m, 2H), 7.446 (m, 2H).

Compound **4** was synthesized in an analogous way, giving a 35% yield of a yellow solid. $^1$H NMR (δ, $CDCl_3$) 0.99 (s, 6H), 2.22 (s, 3H), 6.71 (d, 1 H), 7.28 (s, 1H), 7.33 (d, 1H).

*4.2.11 Synthesis of benzopentathiepins **5** and **6**.* The pentathiepin ring was synthesized as described previously.[25,26] A solution of **3** (0.1 g, 1.0 equiv) in anhydrous $CH_2Cl_2$ (10 mL) was cooled in ice. Sulfur monochloride was added (50 µL, 1.5 equiv) and the reaction stirred for 4 days (monitored by TLC), warming to room temperature. A 0.5 N solution of HCl (10 mL) was added to quench the reaction. The organics were extracted with 3 x 10 mL $CH_2Cl_2$, washed with 1 x 10 mL brine, dried over anhydrous $Na_2SO_4$, and filtered, and the solvent was removed. The crude product was further purified on a silica column, eluting with hexanes to give **5** as a yellow oil in 27% yield. $^1$H NMR (δ, $CDCl_3$) 7.325 (m, 2H), 7.830 (m, 2H). The analogous reaction using **4**

yielded **6** as a yellow oil in 38% yield. $^{1}$H NMR (δ, $CDCl_3$) 2.37 (s, 3H), 7.14 (d, 1H), 7.65 (s, 1H), 7.69 (d, 1H). LC-MS experiments gave no useful data.

*4.2.12 Preliminary studies of $H_2S$-release from 3-methylbenzopentathiepin,* ***6****.* A solution of 3-methylbenzopentathiepin (**6**, 34.17 mg) in $CH_2Cl_2$ (1.36 mL) was previously prepared for mass spectrometry studies. Because of evaporation, the exact concentration of **6** used in this study may not be accurate. A 10 μL aliquot of **6** was diluted further with 90 μL DMSO to form a 10 mM solution. This dilution was used in 200 μL total volume with 2 μL **6** solution (100 μM), 10 μM DMSO used to dissolve AzMC (10 μM), and 5 mM glutathione in buffer (200 mM Tris HCl, 100 mM NaCl, pH 8.0).

## 4.3 Thiuram Disulfides as Potential Hydrogen Sulfide-Releasing Agents

We hypothesized that thiuram disulfides might serve as potential $H_2S$-donating agents. Two such compounds are known drugs, disulfiram and imuthiol. These compounds should, therefore, not be overly toxic as they have been well studied in humans. During an initial screen of a thiuram disulfide library (Figure 4.3), we found that several compounds produced $H_2S$ in the presence of 10 mM glutathione or 250 μM cysteine (Figure 4.4a). Upon countertesting these results using the methylene blue assay and 2 mM glutathione, we confirmed the release of $H_2S$ (Figure 4.4b). While several other thiruam disulfides produce small amounts of $H_2S$ (up to 0.5 equiv), **4C** was by far the greatest producer of $H_2S$. (A note should be made that often less $H_2S$ was seen after 24 h preincubation than when the reaction time was much shorter. We theorize that this is

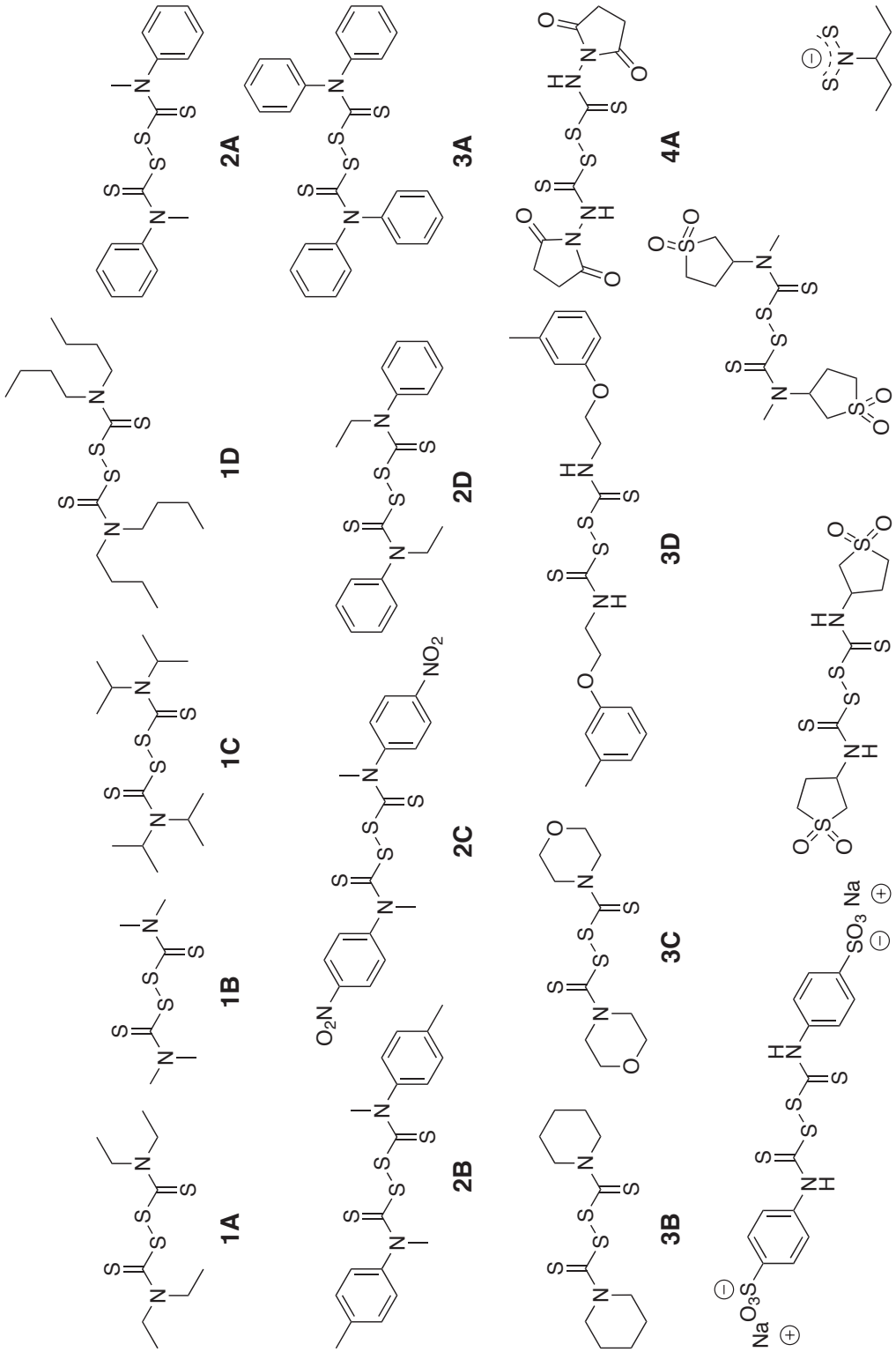



Figure 4.3 Thiuram Disulfiram library. Disulfiram is **1A** and imuthiol is **5A**. The most promising $H_2S$-donor is **4C**.

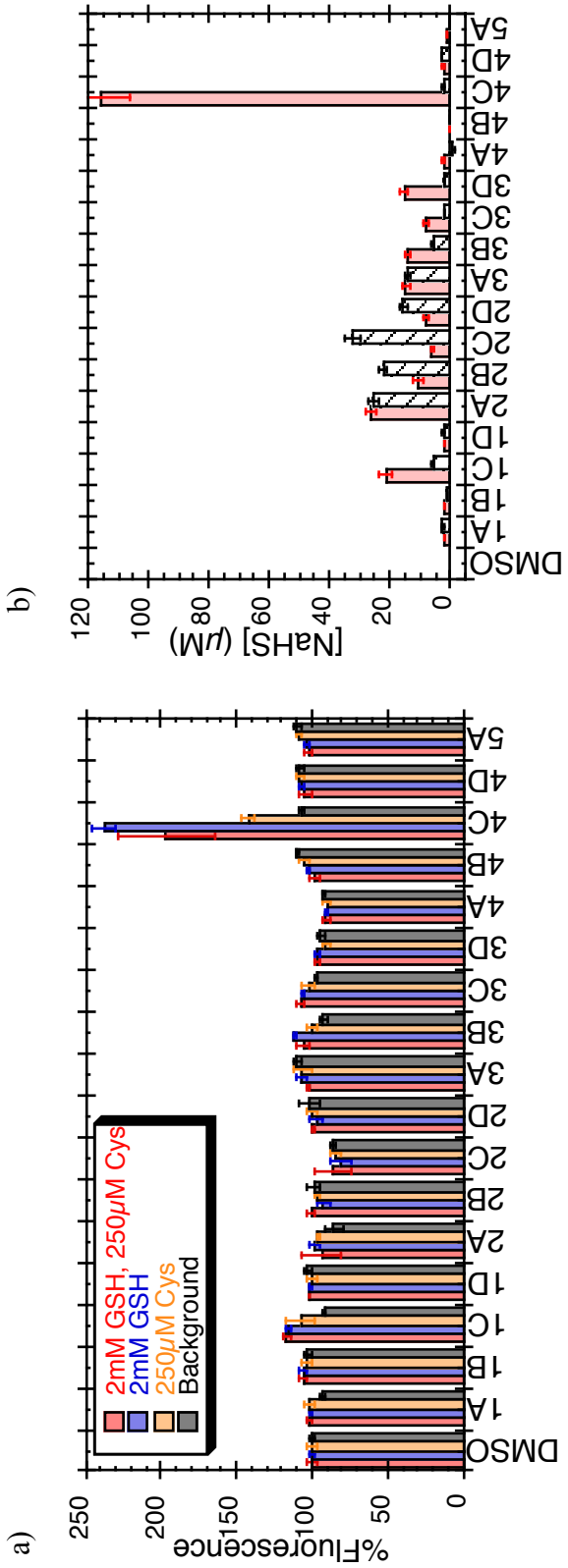



Figure 4.4 Initial screen of thiuram disulfide library. a) Thiuram disulfide (100 μM) response to glutathione (2 mM) and/or cysteine (250 μM) in using the presence of AzMC (10 μM) after 1 h. b). Thiuram disulfides (100 μM) were incubated in the presence (red) or absence (black) 2 mM glutathione for 24 h. H2S was detected using the methylene blue assay.

due to evolution of $H_2S$ into the headspace of the eppendorf tube. Indeed, a higher concentration of $H_2S$ is recorded during experiments with either greater volume or a smaller headspace.) We therefore, will focus on compound **4C** for the remainder of this section.

*4.3.1 Compound **4C** reactivity*. Upon discovery that compound **4C** produces $H_2S$ in the presence of a glutathione, we had two questions: first, what is required for this reaction to take place? And second, why does **4C** produce so much more $H_2S$ than related compounds? To answer the first question, a number of reducing agents were tested (Figure 4.5). We found that $H_2S$ release from **4C** was dependent on reduction of **4C** by a thiol-based reductant. While glutathione, DTT, BME, and cysteine all induced $H_2S$-release from **4C**, the use of TCEP as the reductant resulted in very little $H_2S$ release.

To answer the second question, we compared sulfolane and **4C** (Figure 4.6) in the presence and absence of 2 mM glutathione. Unlike **4C**, sulfolane does not produce a notable amount of $H_2S$ upon the addition of glutathione. In addition to these studies, we

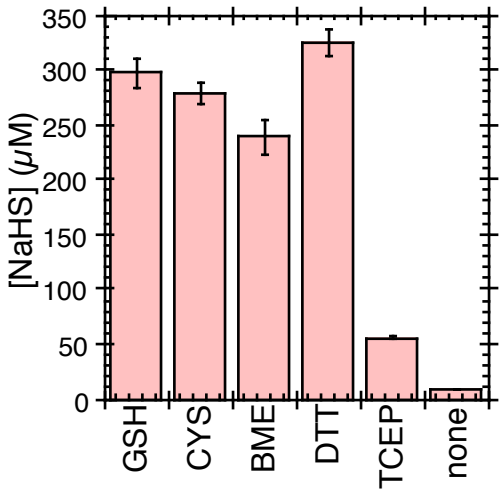



Figure 4.5 Reactivity of **4C** (100 μM) with reducing agents (2 mM) after 30 min incubation. Methylene blue assay used to determine $H_2S$ production.

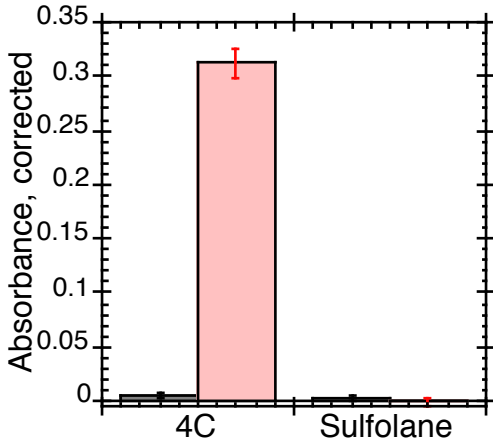



Figure 4.6 Sulfolane (100 μM) control versus **4C** (100 μM) in the presence (red) and absence of (black) 2 mM cysteine.

also investigated the pH dependence of this reaction. We ound that $H_2S$-release was not severely pH dependent (Figure 4.7), but that a slight peak exists at pH 8.0. Lastly, we attempted to quantify the amount of $H_2S$ produced using both the AzMC and methylene blue assays (Figure 4.8). We found that roughly two equivalents of $H_2S$ were produced independent of which assay was used.

*4.3.2 Comparison of **4C** with other $H_2S$-donating compounds.* Having established **4C** as a novel $H_2S$-donating compound, we set out to validate it against two other $H_2S$-donating compounds reported in the literature, DADS and GYY4137. We first compared the response of the three compounds against various reducing agents (Figure 4.9). The first observation of note is that **4C** appears to release the most $H_2S$ over the 2 h time period (GYY4137 is close in the presence of TCEP, although this reaction is not physiologically relevant). As expected, **4C** requires a thiol-based reductant as does, it appears, DADS. In fact, the addition of TCEP to either **4C** or DADS does not stimulate any statistically significant $H_2S$ production. It is interesting that, although GYY4137 is

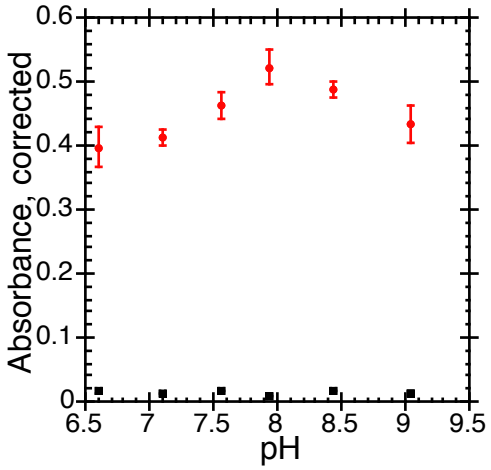



Figure 4.7 pH dependence of $H_2S$ production from **4C** (100 µM) in the presence (red) and absence (black) of cysteine (2 mM) after 30 min incubation. The methylene blue assay was used.

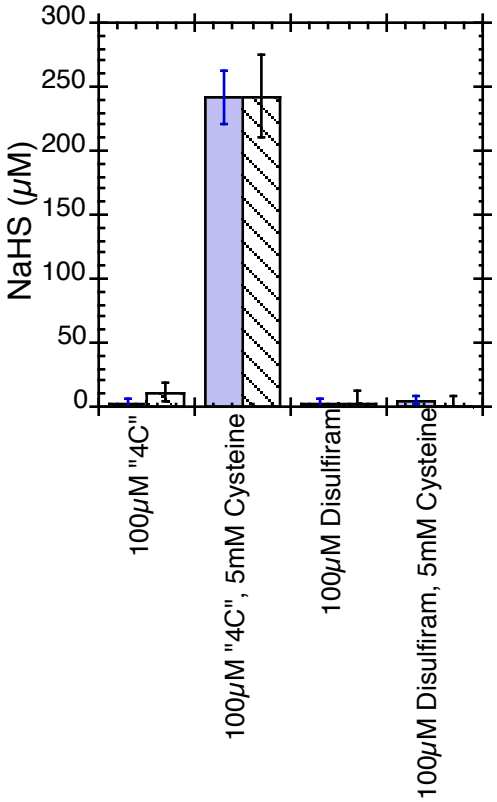



Figure 4.8 Comparison of methylene blue (blue) and AzMC (black) assays. It appears that roughly two equiv of $H_2S$ are produced from **4C** independent of assay used. Disulfiram, **1A**, is used as a control.

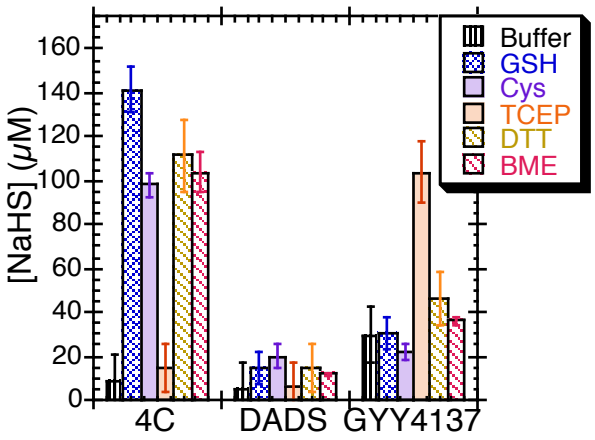



Figure 4.9 Production of $H_2S$ from known $H_2S$-donating compounds (100 μM) in the presence of various reducing agents (5 mM) after 2 h incubation.

reported to react with glutathione and other thiols in red blood cells,[10,17] GYY4137 has the opposite trend as **4C** and DADS in our hands. Under our conditions, GYY4137 does not produce any $H_2S$ upon the addition of glutathione under our conditions and produces very little $H_2S$ in the presence of the other thiol-containing reducing agents. GYY4137 does react quite strongly, however, with the nonthiol reducing agent, TCEP.

Because the rate of $H_2S$ production will be important in biological applications, we also compared the kinetics of $H_2S$ release from DADS, GYY4137, and **4C** (Figure 4.10). In reports, GYY4137 released 4% of all possible $H_2S$ over the first 10 min and another 4% per hour after that for the remaining 80 min at pH 3.0. This same pattern is not seen in our studies at pH 8.0. Instead, we see a quick release of $H_2S$ before the plate is read ( < 30 s) with no subsequent release over the following 2 h. DADS follows a similar pattern. However, $H_2S$-release from **4C** is gradual over the first 40 min before tapering off.

*4.3.3 Compound **4C** structure determination*. We were excited to identify a potent

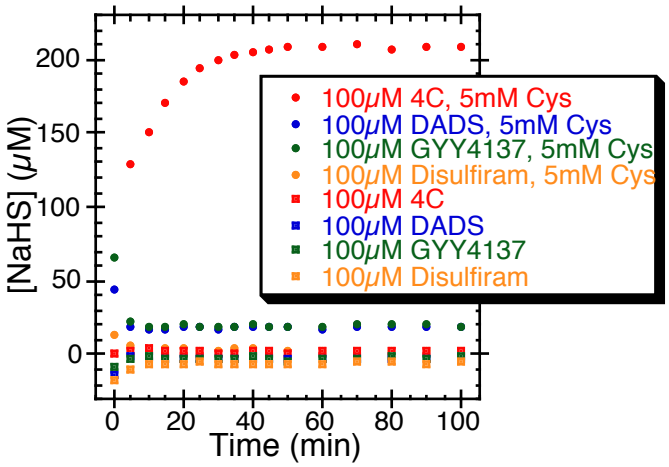



Figure 4.10 Kinetic response of 100 μM **4C** (red), DADS (blue), GYY4137 (green), or Disulfiram (orange) to 5 mM cysteine.

new $H_2S$-donating compound. Hoping that it would also explain the structure-activity relationship of our initial screening results, we set out to determine the mechanism by which $H_2S$ is released from **4C**. While the $^1H$ NMR spectrum was consistent with the proposed structure of **4C** (Figure 4.11), mass spectral analysis of **4C** alone indicated the absence of the expected mass (419.94). Instead, only two unidentified masses were seen: 312.00 and 334.98. We theorized that rather than the thiuram disulfide, **4C** could, instead, be a thiourea. In fact, a thiourea library was later screened, but yielded no $H_2S$-donating leads (Figure 4.12).

After extensive trial and error, **4C** was purified on a silica column, yielding two pure compounds, one with the observed masses of 312.00 and 334.98, which we hypothesize to be the thiourea (Figure 4.11), and another with the lower mass of 279.09. Upon testing of each of these pure compounds, neither produced $H_2S$ in the presence of glutathione.

It came to our attention that inorganic sulfur ($S_8$) could be the $H_2S$-releasing

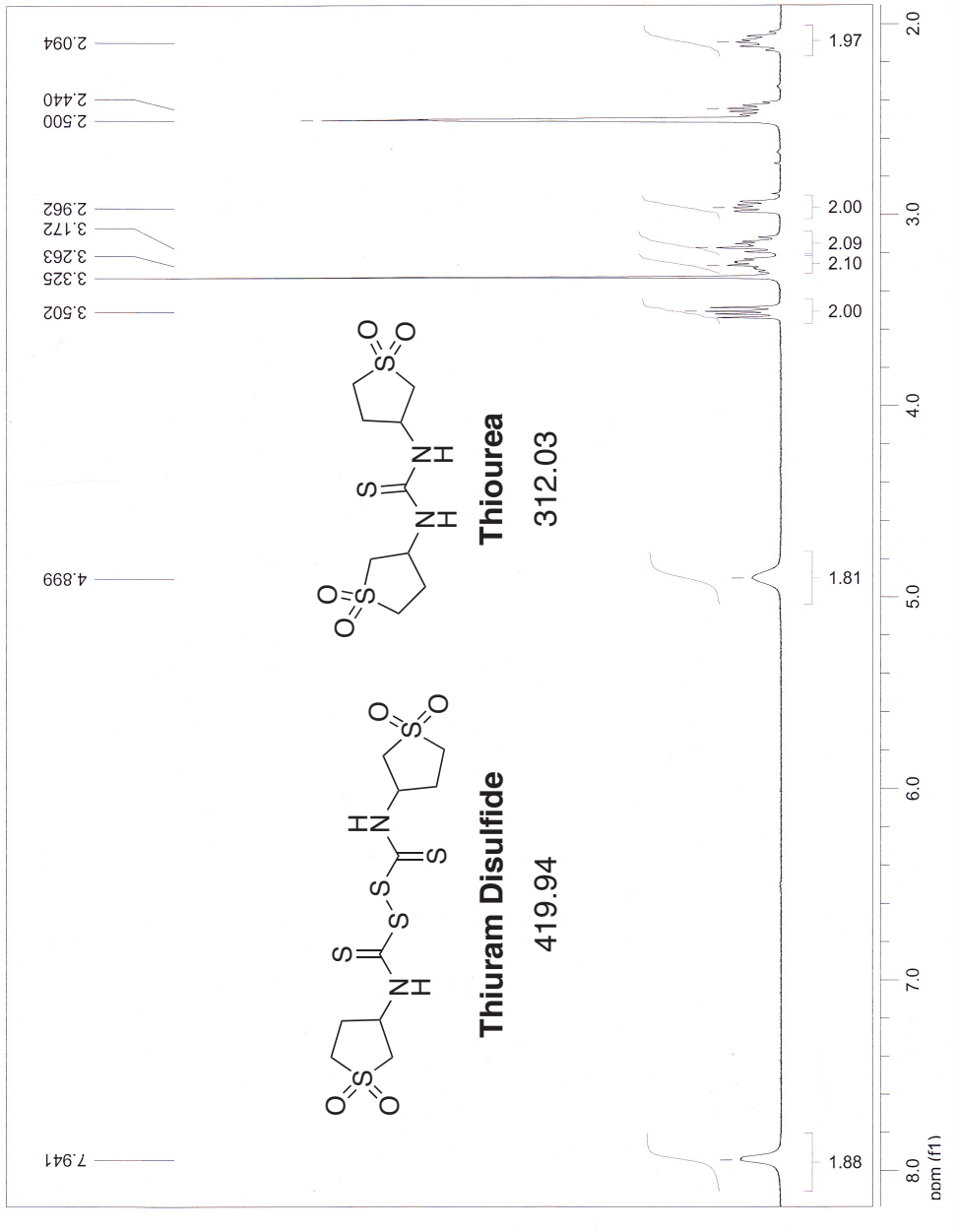



Figure 4.11 $^{1}$H NMR data of commercially purchased **4C** with thiuram disulfide and thiourea structures. Numbers below structures are theoretical exact masses for each.

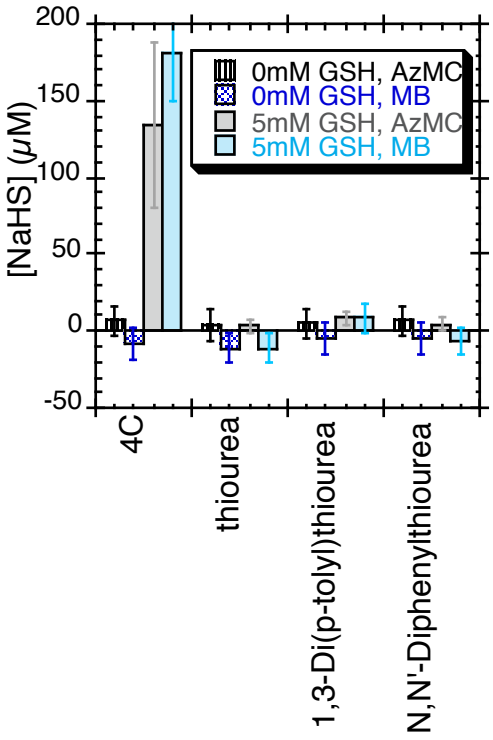



Figure 4.12 Various thiourea derivatives (100 μM) are unable to produce $H_2S$ in the presence of reducing agents, while **4C** does form $H_2S$ in both assays. Reaction was incubated for 1 h.

aqueous or organic solvents, examination of the liquor when suspended in DMSO gave the same results as **4C**. In fact, addition of 100 mM BME to the liquor gave a 25 mM NaHS-equivalent solution that was later used in experiments with crude oil (Chapter 5).

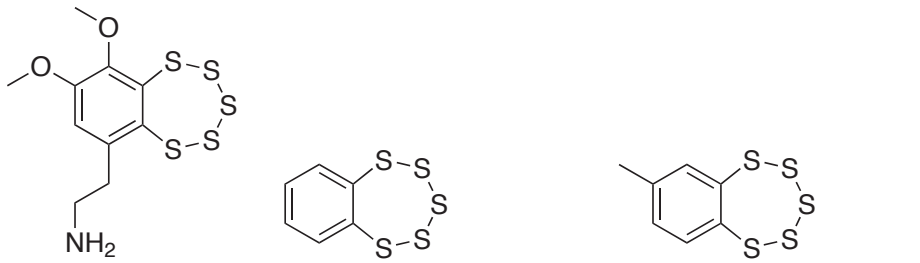
### 4.4 Benzothiepins as Hydrogen Sulfide-Releasing Agents

Based on our experience with inorganic sulfur, knowledge of DADS, and recent reports indicating the biological importance and relevance of polysulfides and persulfides,[23,28] we hypothesized that an organic polysulfide might have utility as a reduction-labile, $H_2S$-donating therapeutic. Our collaborators in the Ireland lab identified

such a compound in 1991, varacin (Figure 4.13).[29] Varacin is a unique benzopentathiepin with antitumor properties.[24,30,31] While the synthesis of varacin has since been established,[29,31] it is not overly straightforward nor high-yielding. We therefore opted to begin out studies with a simpler benzopentathiepin system (Figure 4.13).

*4.4.1 Synthesis of benzopentathiepins.* The synthesis of both benzopentathiepin (**5**) and 3-methylbenzopentathiepin (**6**) are described pictorially in Figure 4.2. There are two major protocols for establishing the pentathiepin ring. The most common procedure involves the use of $S_8$ in neat ammonia. For reasons described above, we chose to avoid the risk of an $S_8$ contaminant and chose instead to go through a dimethyltin intermediate. We first activated the dithiol starting material using dimethyltin chloride. The pentathiepin ring was then inserted using sulfur monochloride ($S_2Cl_2$). Extraction into organic solvent gave the product in 90% purity; however, because pure compounds were essential to our experiments, a silica column was used to further purify each compound.

*4.4.2 Preliminary studies of 3-methylbenzopentathiepin*. Before further validating the structure of compounds **5** and **6**, we were interested to know if they were capable of producing $H_2S$. We began by studying **6,** which proved insoluble in polar solvent.




**Varacin** Benzopentathiepin **5** 3-methylbenzopentathiepin **6**

Figure 4.13 Comparative structures of the natural product varacin and two synthetic benzopentathiepins.

Therefore a stock solution was made using $CH_2Cl_2$ and further diluted in DMSO before experiments were conducted. Compound **6** alone does not release hydrogen sulfide in buffer; however, upon addition of 5 mM glutathione, the evolution of $H_2S$ can be seen over the first 30 min (Figure 4.14). This supports our hypothesis that these pentathiepins, like other polysulfides, may be useful $H_2S$-donating compounds.

*4.4.3 Future work.* Validation of **5** and **6** remains to be done. Along with a more detailed NMR study, mass spectroscopy and elemental analyses will be used to confirm the addition of three sulfur atoms to the parent dithiols (the addition of one sulfur atom to make a five-membered ring or five sulfur atoms to make a nine-membered ring could also be expected). Additionally, solubility in aqueous solution is desirable. Therefore, a more polar benzopentathiepin should be designed, synthesized, and tested.

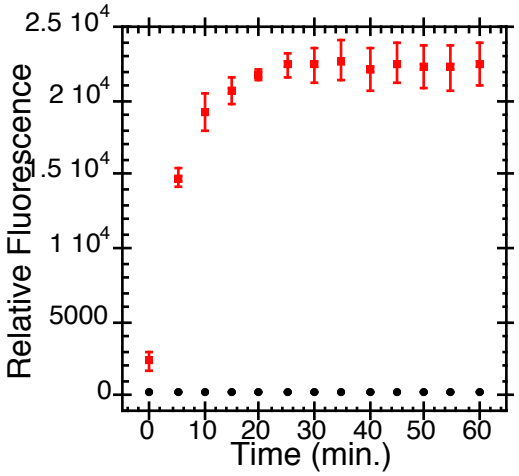



Figure 4.14 Preliminary studies of **6** (~100 μM) in the presence (red) and absence (black). 5 mM glutathione shows production of $H_2S$.

## 4.5 Conclusion

Because of the important functions of hydrogen sulfide and other reactive sulfur species in biology, $H_2S$-donating compounds may have great value. These agents could be used to further investigate the production of $H_2S$ *in vivo*. They could also potentially have use as therapeutics in the treatment of numerous $H_2S$-associated diseases.[17,32] To this end, we have investigated a new $H_2S$-donating compound, **4C**. Though the exact $H_2S$-releasing contaminant in **4C** remains a mystery, the conditions required to produce $H_2S$ from **4C** have been thoroughly investigated using both fluorescent and colorimetric assays. It has been established that this contaminating compound is reduction-labile (such as a persulfide, polysulfide, or inorganic sulfur) and produces a controlled release of $H_2S$ over 40 min in the presence of a reducing thiol.

Two polysulfides based on the natural product varacin have been designed and synthesized. Preliminary evidence suggests that these benzopentathiepin compounds release $H_2S$ over a 20-min interval upon exposure with thiol-based reducing agents. These compounds show potential as novel $H_2S$-donating compounds and will hopefully lead to more benzopentathiepin-based agents with improved solubility.

## 4.6 References

(1) Ishigami, M.; Hiraki, K.; Umemura, K.; Ogasawara, Y.; Ishii, K.; Kimura, H. *Antioxid. Redox Signal.* **2009**, *11*, 205–214.

(2) Ichinohe, A.; Kanaumi, T.; Takashima, S.; Enokido, Y.; Nagai, Y.; Kimura, H. *Biochem. Biophys. Res. Commun.* **2005**, *338*, 1547–1550.

(3) Eto, K.; Asada, T.; Arima, K.; Makifuchi, T.; Kimura, H. *Biochem. Biophys. Res. Commun.* **2002**, *293*, 1485–1488.

(4) Zhang, M.; Shan, H.; Wang, T.; Liu, W.; Wang, Y.; Wang, L.; Zhang, L.; Chang, P.; Dong, W.; Chen, X.; Tao, L. *Neurochem. Res.* **2013**, *38*, 714–725.

(5) Yan, J.; Teng, F.; Chen, W.; Ji, Y.; Gu, Z. *Exp. Ther. Med.* **2012**, *4*, 832–838.

(6) Kazuho, A.; Kimura, H. *J. Neurosci.* **1996**, *16*, 1066–1071.

(7) Blom, H. J.; Smulders, Y. *J. Inherit. Metab. Dis.* **2011**, *34*, 75–81.

(8) El-Sayed, A. S.; Shindia, A. A. In *Targets in Gene Therapy*; **2010**; 119–146.

(9) Polhemus, D. J.; Lefer, D. J. *Circ. Res.* **2014**, *114*, 730–737.

(10) Li, L.; Whiteman, M.; Guan, Y. Y.; Neo, K. L.; Cheng, Y.; Lee, S. W.; Zhao, Y.; Baskar, R.; Tan, C.-H.; Moore, P. K. *Circulation* **2008**, *117*, 2351–2360.

(11) Benavides, G. a; Squadrito, G. L.; Mills, R. W.; Patel, H. D.; Isbell, T. S.; Patel, R. P.; Darley-Usmar, V. M.; Doeller, J. E.; Kraus, D. W. *Proc. Natl. Acad. Sci.* **2007**, *104*, 17977–17982.

(12) Rashid, S.; Heer, J. K.; Garle, M. J.; Alexander, S. P. H.; Roberts, R. E. *Br. J. Pharmacol.* **2013**, *168*, 1902–1910.

(13) Chan, M. V; Wallace, J. L. *Am. J. Gastrointest. Liver Physiol.* **2013**, 467–473.

(14) Teague, B.; Asiedu, S.; Moore, P. K. *Br. J. Pharmacol.* **2002**, *137*, 139–145.

(15) Wallace, J. L. *Antioxid. Redox Signal.* **2010**, *12*, 1125–1133.

(16) Olson, K. R. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2011**, *301*, 297–312.

(17) Kashfi, K.; Olson, K. R. *Biochem. Pharmacol.* **2013**, *85*, 689–703.

(18) Kashfi, K. *Antioxid. Redox Signal.* **2014**, *20*, 831–846.

(19) Powolny, A. A.; Singh, S. V. *Cancer Lett.* **2008**, *269*, 305–314.

(20) Ali, M.; Thomson, M.; Alnaqeeb, M. A.; Al-Hassan, J. M.; Khater, S. H.; Gomes, S. A. *Prostaglandins, Leukot. Essent. Fat. Acids* **1990**, *41*, 95–99.

(21) Insko, M. A.; Deckwerth, T. L.; Hill, P.; Toombs, C. F.; Szabo, C. *Br. J. Pharmacol.* **2009**, *157*, 944–951.

(22) Li, H.; Li, H.; Wang, Y.; Xu, H.; Fan, W.; Wang, M.; Sun, P.; Xie, X. *Chin. Med. J. (Engl).* **2004**, *117*, 1155–1160.

(23) Ida, T.; Sawa, T.; Ihara, H.; Tsuchiya, Y.; Watanabe, Y.; Kumagai, Y.; Suematsu, M.; Motohashi, H.; Fujii, S.; Matsunaga, T.; Yamamoto, M.; Ono, K.; Devarie-Baez, N. O.; Xian, M.; Fukuto, J. M.; Akaike, T. *Proc. Natl. Acad. Sci.* **2014**, *111*, 7606–7611.

(24) Lee, A. H. F.; Chen, J.; Liu, D.; Leung, T. Y. C.; Chan, A. S. C.; Li, T. *J. Am. Chem. Soc.* **2002**, *125*, 13972–13973.

(25) Sato, R.; Fujio, T.; Nakajo, S.; Ogawa, S.; Alam, A. *Tetrahedron Lett.* **2007**, *48*, 3013–3016.

(26) Konstantinova, L. S.; Rakitin, O. a; Rees, C. W. *Chem. Rev.* **2004**, *104*, 2617–2630.

(27) Xu, J.; Chatterjee, M.; Baguley, T. D.; Brouillette, J.; Kurup, P.; Ghosh, D.; Kanyo, J.; Zhang, Y.; Seyb, K.; Ononenyi, C.; Foscue, E.; Anderson, G. M.; Gresack, J.; Cuny, G. D.; Glicksman, M. A.; Greengard, P.; Lam, T. T.; Tautz, L.; Nairn, A. C.; Ellman, J. A.; Lombroso, P. J. *PLoS Biol.* **2014**, *12*, e1001923.

(28) Bailey, T. S.; Zakharov, L. N.; Pluth, M. D. *J. Am. Chem. Soc.* **2014**, *136*, 10573–10576.

(29) Davidson, Bradley, S.; Molinski, T. F.; Barros, L. R.; Ireland, C. M. *J. Am. Chem. Soc.* **1991**, *113*, 4709–4710.

(30) Ford, P. W.; Narbut, M. R.; Belli, J.; Davidson, B. S. *J. Org. Chem.* **1994**, *59*, 5955–5960.

(31) Toste, F. D.; Still, I. W. J. *J. Am. Chem. Soc.* **1995**, *117*, 7261–7262.

(32) Szabó, C. *Nat. Rev. Drug Discov.* **2007**, *6*, 917–935.

CHAPTER 5

# LANTHANIDE COMPLEXES AND COLORIMETRIC DYES AS PROBES FOR HYDROGEN SULFIDE WITH INDUSTRIAL AND ENVIRONMENTAL APPLICATIONS*

## 5.1 Introduction

Hydrogen sulfide ($H_2S$) is a gas notorious for its noxious smell and toxicity. As emphasized in pervious chapters, $H_2S$ plays key roles in cellular signaling pathways and may be critical for maintaining vascular and neurological health.[1] However, hydrogen sulfide is also ubiquitous in the environment. This gaseous molecule is a key contaminant in crude oil[2] and ground water[3] and is an important cause of work-related sudden death.[4] Due to the industrial, environmental and biological importance of $H_2S$ and related sulfides, there has been significant interest in the development of chemical approaches for monitoring its presence.

Described in greater detail in Chapter 1, traditional approaches to hydrogen sulfide detection include the methylene blue assay first reported by Emil Fischer in the late 1800s,[5] qualitative analysis of sulfide by precipitation with lead acetate, the use of a

* This work is an adaptation of a paper previously prepared for publication. Thorson, M. K.; Ung, P.; Tuck, K. L.; Graham, B.; Barrios, A. M. *manuscript in preparation*.

sulfide ion selective electrode, and gas chromatography.[6] Due to the recent interest in the biological roles of $H_2S$, a number of fluorogenic probes for $H_2S$ have also been developed.[7–9] These probes generally rely on sulfide-specific chemistries; most notably the sulfide-selective reduction of an aryl azide or nitro group to form an aryl amine with concomitant increase in a fluorescent signal.[10,11] While these reaction-based probes offer significant advantages over other approaches to monitoring $H_2S$, including a sensitive fluorogenic signal and a direct and facile assay for the presence of $H_2S$, they have not been widely applied to industrially or environmentally relevant samples. In addition, the excitation and/or emission profiles often overlap with small molecule absorbance and fluorescence and tissue autofluorescence.[12,13] One way to get around this difficulty is to utilize fluorogenic probes with long luminescent lifetimes[14] that allow for time-gated fluorescence studies. By allowing the background (organic) fluorescence to decay before a signal is read, the signal-to-noise ratio is improved greatly. This enables these probes to have utility in scenarios where other fluorescent probes may fail to produce a reliable signal.

## 5.2 Materials and Methods

*5.2.1 General considerations.* All chemicals were purchased from commercial sources and used as received unless indicated otherwise. The $^1H$ NMR data were collected on a Varian 400 MHz NMR spectrometer. Chemical shifts are reported in parts per million referenced to internal standard $(CH_3)_4Si$ = 0.00 ppm. Fluorescence and UV/Vis data were collected using a Molecular Devices Spectra Max M5 plate reader. Lanthanide probes were supplied by the laboratory of Dr. Bim Graham at Monash

Institute of Pharmaceutical Sciences.

*5.2.2 Screening of lanthanide-based probes.* Eight lanthanide probes containing an azide functional group were screening for reactivity with hydrogen sulfide. Probe fluorescence was determined both with and without 100μM NaHS in buffer (200 mM Tris HCl, pH 8). Time-resolved fluorescence was performed using a 500 μs delay and integrating over 1000 μs. Probes were excited at both 270 and 360 nm. All probes were soluble in buffer with the exception of probe 3, which was dissolved in DMSO.

*5.2.3 NaHS dependence of compounds 1, 2, and 3.* A final concentration of 10 μM of each compound was used in buffer (200 mM Tris HCl, pH 8.0) and 5% DMSO. Optimal excitation of each probe was determined by observing the emission fluorescence at 545 nm or 615 nm while varying the excitation wavelength both in the absence and presence of 250 μM NaHS (delay: 500 μs, integration: 1000 μs).

*5.2.4 Measuring hydrogen sulfide levels in sour and stripped water.* Known concentrations of NaHS (0–300 μM) were created and used to make standard curves for all three assays. The sour water was diluted sequentially to create a dilution within the workable range of all assays. For the compound 3 and AzMC, 10 μM of probe in buffer and 5% DMSO was incubated with known NaHS solution for 30 min prior to detection. Standard methylene blue protocol was used for validation.[15–17] Briefly, a 70 μL aliquot of known NaHS concentration was reacted with 60 μL 10% w/v trichloroacetic acid, 30 μL 1% w/v zinc acetate (aq), and 20 μL 30 mM ferric chloride (in 1.2 M HCl). Addition of 20 μL 20mM *N,N*-dimethyl-*p*-phenylenediamine sulfate (in 7.2 M HCl) yielded a blue color, which was detected after 30 min at 670 nm.

*5.2.5 Initial studies with compound **1** in crude oil #4.* The 200 μL total solution

was composed of 10 μL buffer, 10 μL 0.2 mM probe in DMSO (10 μM final concentration), and either 10 μL "control" or "sulfide." Because of limited NaHS solubility in buffer, an alternative form of hydrogen sulfide was used for these initial experiments. The liquor of inorganic sulfur pieces suspended in DMSO was combined with β–mercaptoethanol (BME) (7 μL BME in 1 mL total solution) to produce "sulfide." To control for the DMSO and BME, a "control" was added to nonsulfide containing wells. The "control" solution was identical to the "sulfide," but contained pure DMSO. The "sulfide" solution was later determined to have a concentration equal to that of 25 ±3 mM NaHS using the AzMC assay in buffer. The solutions were incubated for 30 min prior to fluorescence determination. Maximal emission (615 nm) was seen at an excitation of 360 nm after a 500 μs delay and 1000 μs integration.

*5.2.6 Initial studies with compound **3** in crude oil #4.* The 200 μL total solution was composed of 10 μL buffer, 50 μL 0.2 mM probe in DMSO (10 μM final concentration) and either 50 μL "control" or "sulfide." See 5.2.5 for solution definitions. Compound 3 gives a maximal emission at 545 nm when excited at 280 nm during time-resolve fluorescence studies.

*5.2.7 NaHS dependence of compounds **1** and **3** in crude oil #4.* The response of compound 1 in crude oil was determined in an analogous manner to that described in section 5.2.6. A 10 μL aliquot of probe (10 μM final concentration) in DMSO was added to 10 μL crude oil and 10 μL buffer in dioxane. The same experiment was conducted without oil as a control. Compound 1 was excited at 360 nm and the emission was read at 615 nm following a 500 μs delay and 1000 μs integration. Compound 3 was excited at 280 nm to give a signal at 545 nm.

*5.2.8 Synthesis of 4-azidoazobenzene, **4**.* 4-azidoazobenzene was synthesized in an analogous fashion to our previous azide-based probes. Aniline yellow (1.2 g, 1.0 equiv) was suspended in water (25 mL) and allowed to stir at 0 °C in an ice/water bath. Concentrated sulfuric acid (6 mL) was added to the reaction in a dropwise fashion to maintain the temperature. Sodium nitrate (0.55 g, 1.3 equiv) was dissolved in water (7 mL), cooled to 0 °C, and added in a dropwise fashion to the reaction over 10 min. The reaction was then allowed to stir at 0 °C for 1 h. Meanwhile, sodium azide (0.67 g, 1.6 equiv) was dissolved in water (5 mL) and cooled to 0 °C. After 1 h, the sodium azide solution was added to the reaction mixture in a dropwise fashion, and the formation of a red-orange precipitate was immediately observed. The reaction mixture was then allowed to warm to room temperature overnight with stirring, and the solid was collected using vacuum filtration and washed with water (150 mL). The resulting solid was then dissolved in chloroform (200 mL) and ethyl acetate (100 mL), dried over anhydrous magnesium sulfate, and filtered, and the solvent was removed to yield a dark powder. The powder was dissolved in ethyl acetate (40 mL) and washed with 3 x 20 mL 0.5 N HCl, and the organic layer was further washed with 2 x 20 mL of a brine solution. The organic layers were collected, dried over anhydrous sodium sulfate, and concentrated. The concentrated solution was purified on a silica column, eluding with 1:5 ethyl acetate: hexane before being further purified on a silica column eluding with hexanes. The resulting solid was the pure 4-azidoazobenzene (0.917 g) in 65.5% yield. $^{1}$H NMR (δ, $(CD_3)_2SO$) 7.34 (d, 2H), 7.58 (m, 3H), 7.87 (d, 2H), 7.94 (d, 2H).

*5.2.9 Reactivity of 4-azidoazobenzene.* A 100 µL aliquot of 4-azidobenzene was reacted with NaHS (0–20 mM) in 200 µL total volume buffer with 5% DMSO used to

dissolve the probe. No response was seen between 7-aminoazobenzene and NaHS.

## 5.3 Results and Discussion of Lanthanide-Based Hydrogen Sulfide Probes

*5.3.1 Lanthanide-based probes and their reactivity*. After obtaining a small series of lanthanide-based aryl azides from the Graham lab, each was studied for reactivity with NaHS. During the course of our work, compound **1** was reported in the literature as a $H_2S$-selective probe,[18] showing a linear response to $H_2S$ in the concentration range between 80 nM and 1 μM.[18] Of the eight compounds tested, three displayed a response upon the addition of 250 μM NaHS (Figure 5.1).

The emission profiles of each compound in the absence and presence of 250 μM NaHS are displayed in Figure 5.2. Compounds **1** and **3**, upon excitation at 280 nm and 350 nm, respectively, display increased fluorescence in the presence of NaHS. These compounds, especially compound **3**, with an approximately 5-fold fluorescence increase, should be good "turn-on" fluorescent probes for $H_2S$. Upon excitation at 280 nm, compound **2** displays decreased fluorescence in the presence of $H_2S$ and can be considered a "turn-off" probe of $H_2S$. Additionally, the $\lambda_{ex}$ values for compounds **1** and **2**

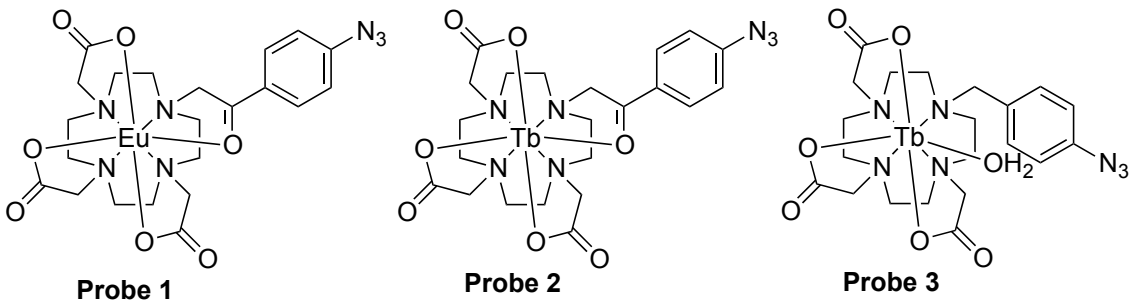



Figure 5.1 Structures of $H_2S$-responsive lanthanide compounds.

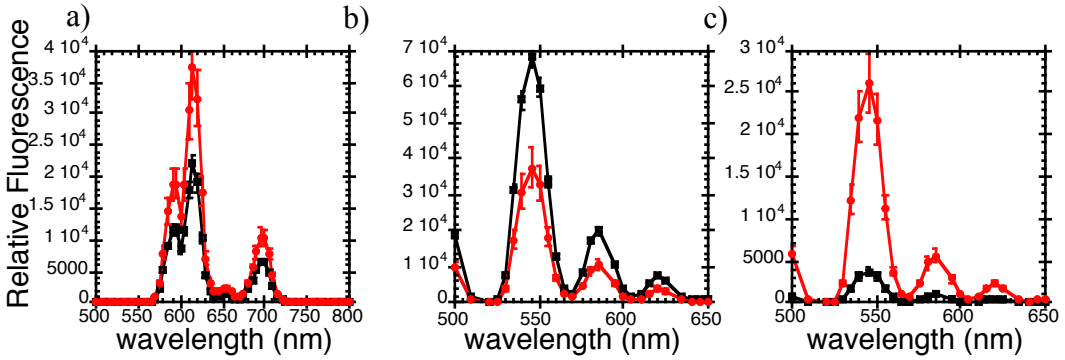



Figure 5.2 Response of compounds **1** (a), **2** (b), and **3** (c) with 250 µM NaHS. Closed circles (red) indicate the presence of NaHS while open squares (black) represent background fluorescence in the absence of sulfide.

are significantly red-shifted as compared to that of compound **3**, with significant absorbance above 300 nm. In contrast, compound **3** has little absorbance above 300 nm (Figure 5.3).

While compounds **1** and **3** both have the potential to serve as $H_2S$ sensors with significant time-delayed luminescence, the sensitivity of the compound along with their excitation profiles will likely influence their utility as probes. As shown in Figure 5.4, compound **3** could reliably detect NaHS in concentrations as low as 250 nM in aqueous solutions with a linear working range from 250 nM to 300 µM NaHS. Because of its sensitivity, large working range, and UV excitation, this compound was predicted to have good utility in measuring $H_2S$ levels in industrial water samples with little background absorbance.

*5.3.2. Hydrogen sulfide detection in sour and stripped water.* Large amounts of water are used in the process of refining crude oil, and the industry generates significant volumes of wastewater.[19] This wastewater (often called "sour water" because of its characteristic smell) contains large amounts of sulfide, ammonia, and other petroleum

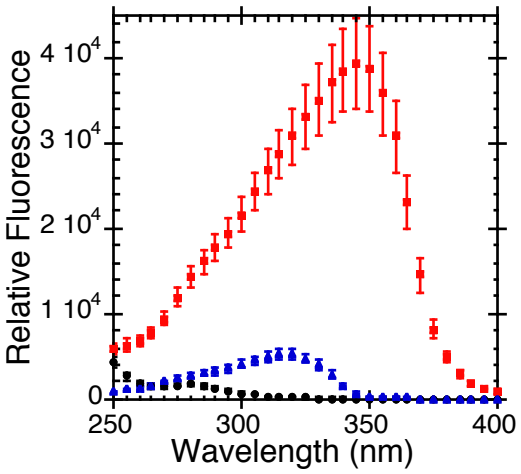



Figure 5.3 Excitation sweep of 10 µM compound **1** (blue), **2** (red), and **3** (black) in the presence of 250 µM NaHS, emission read at 615 nm.

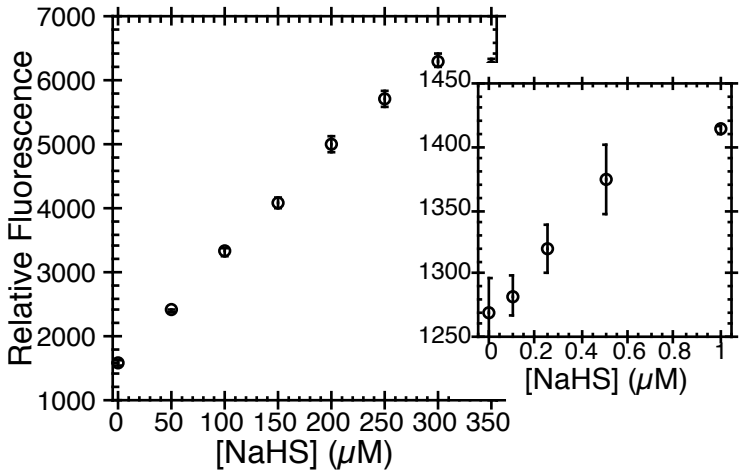



Figure 5.4 Concentration dependence of NaHS on compound **3**.

byproducts. Before the water can be released into the environment or recycled in the refinery, it must be "stripped" of the toxic and corrosive byproducts. Specifically, the Environmental Protection Agency stipulates that the hydrogen sulfide in water released into the environment must be below the olefactory detection limit (estimated at approx. 15 μM).[14] To validate the utility of compound **3** for detecting hydrogen sulfide in aqueous samples, we obtained samples of "sour" and "stripped" water from a local oil refinery. Using the standard curve shown in Figure 5.4, we measured the concentration of $H_2S$ in our industrial water samples (Figure 5.5). The sour water sample had a very high sulfide concentration of 40 ±4 mM as measured by probe **3**. This measurement was independently verified using two proven quantitative tests for $H_2S$, the methylene blue test and the fluorescent probe AzMC.[5,20] The stripped water sample had a significantly lower sulfide concentration of 3.0 ±0.4 μM as measured using probe **3** and verified using

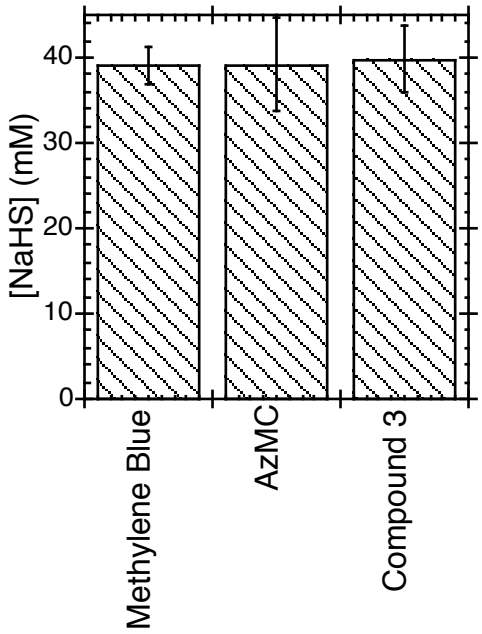



Figure 5.5 Detection of hydrogen sulfide in sour water using three independent assays.

our coumarin-based probe, AzMC. The methylene blue method is not sensitive below 10 μM and therefore could not be used to further validate these results.[21]

*5.3.3 Detection of hydrogen sulfide in crude oil.* While hydrogen sulfide levels were readily detected in sour water, the detection of hydrogen sulfide in crude oil posed more challenging. First, crude oil has considerable background fluorescence, a result of many contaminating hydrocarbons. This obstacle is easily overcome by use of time-resolved fluorescence studies as previously described. Second, crude oil has low light transmission, especially at wavelengths below 300 nm (Figure 5.6). Low wavelengths are absorbed by the oil sample, making it extremely difficult to excite a probe. Lastly, crude oil, understandably, is not readily soluble in aqueous solutions. The reduction of azide-based probes by hydrogen sulfide, however, is a water dependent reaction, something difficult to reconcile within a crude oil sample.

Using dioxane as a solvent for its ability to dissolve both oil and water, we began our studies with compound **3**. We first attempted to detect hydrogen sulfide in the

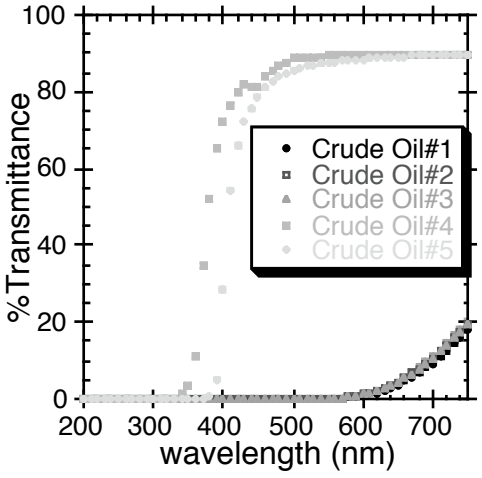



Figure 5.6 Uv/Vis spectra of 100 μL crude oils 1–5 obtained from the Tesoro Corporation Refinery in Salt Lake City, Utah.

presence of 5% crude oil #4. While a significant increase in signal was seen in dioxane alone, the addition of oil decreased this signal drastically (Figure 5.7). We therefore opted to change our focus to compound **1**. Compared to compound **3**, compound **1** has the advantage of a higher excitation wavelength and is compatible with industrial oil samples, although it is not as sensitive in buffer. We theorized that this compound would prove more useful in samples with a high background absorbance and fluorescence. Indeed, a significant increase was seen in the presence of crude oil #4 (Figure 5.7) as a result of the higher excitation wavelength. In fact, compound **1** was able to detect added NaHS in the darkest crude oil samples, oils #1–3.

After validating the usefulness of compound **1** in crude oil, we also tested the linear working range for compound **1** in 5% crude oil #4 with additional NaHS. We

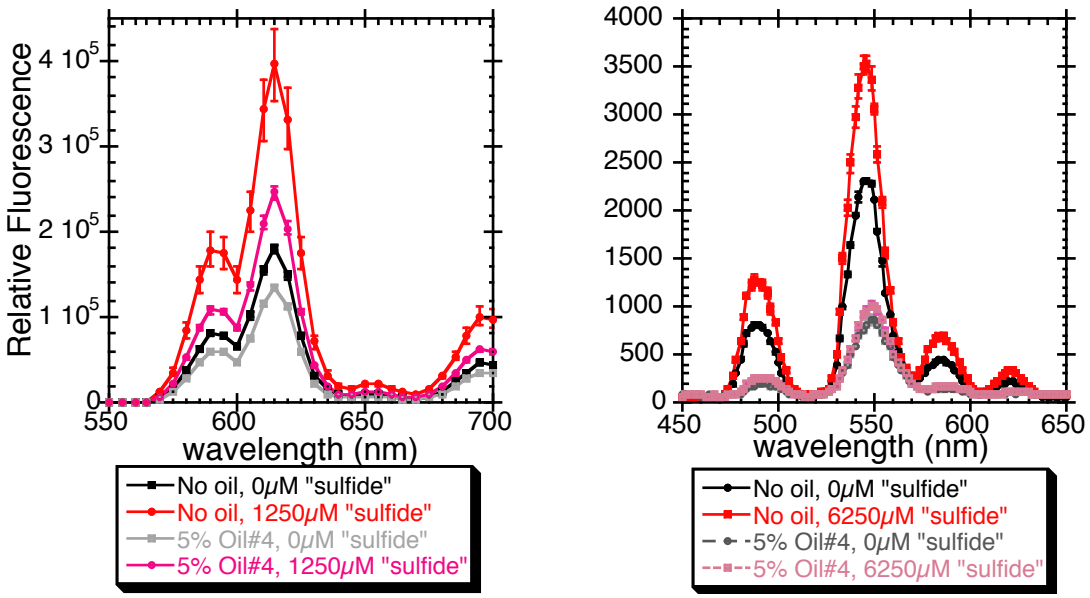



Figure 5.7 Lanthanide complexes as $H_2S$ probes in crude oil. Left, compound **1** in the presence (solid) and absence (open) of oil # 4, with (red/pink) and without (black/grey) sulfide. Right panel, repeated with compound **3**. A larger concentration of sulfide was needed.

found the range to be similar to that of compound **3** in buffer; namely, additional NaHS was detected from 50–300 μM (Figure 5.8).

### 5.4 Results and Discussion of Colorimetric Hydrogen Sulfide Probes

As a complement to the fluorescent probes, a colorimetric hydrogen sulfide-selective probe was also desired. Theoretically, a probe with a strong visual response would have use not only in industrial laboratories but also to wider communities. While only specialists possess the proper instrumentation to detect hydrogen sulfide via a fluorescent signal, a visual change, for example from colorless to pink, can be readily detected by the majority of the population. Such probes could be used to detect hydrogen sulfide contaminating ground or drinking water, or they could have use in the petrochemical industry as part of a two-phase assay. To this end, we have prepared the azide-derivative of aniline yellow, 4-azidoazobenzene (Figure 5.9), as a potential colorimetric probe for $H_2S$.

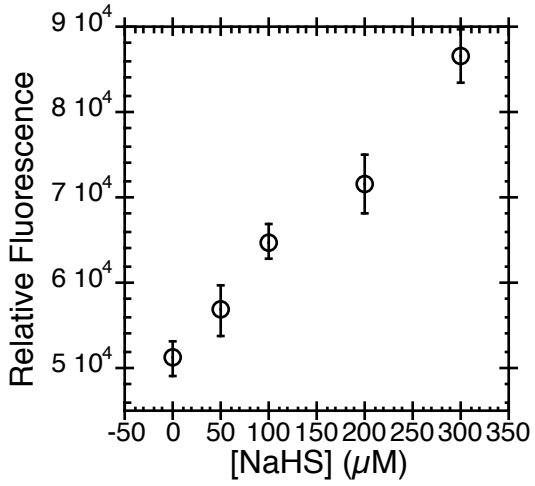



Figure 5.8 NaHS concentration dependence of 10 μM compound **1** in the presence of 5% crude oil #4 in dioxane

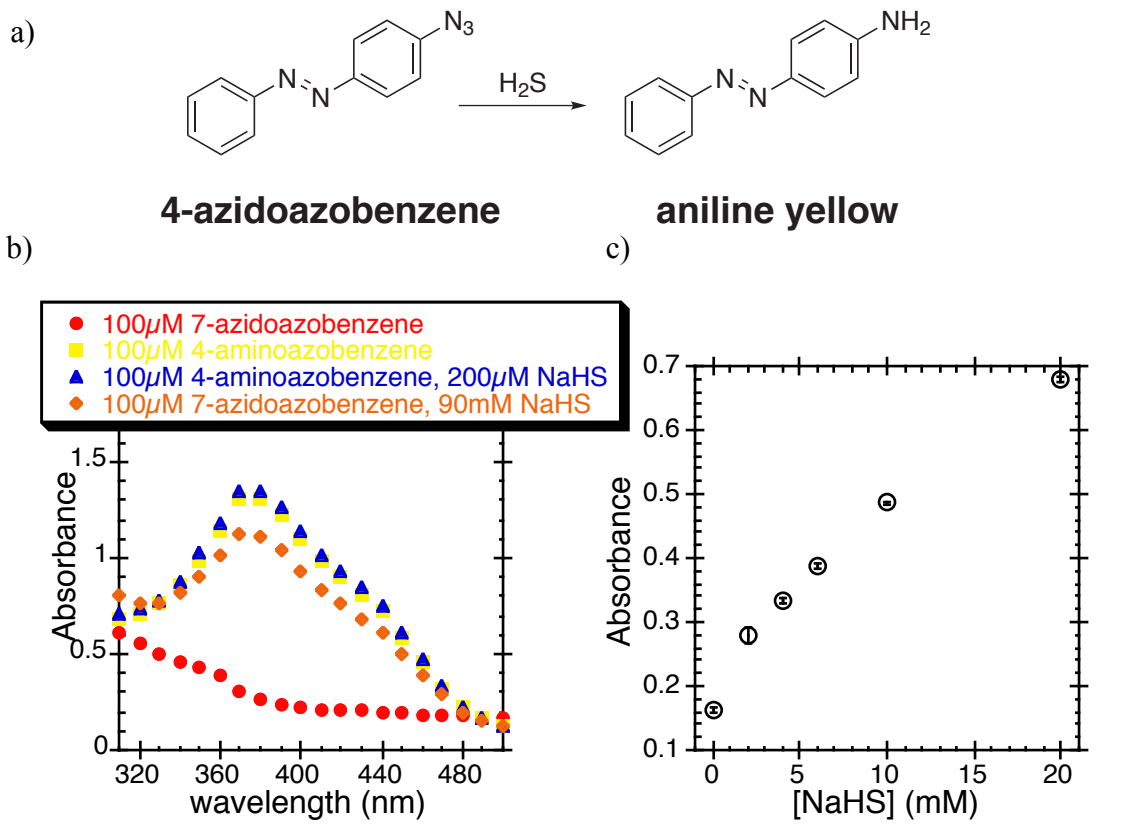



Figure 5.9 Studies of 7-Azidoazobenzene. a) Aniline yellow is produced by reduction of 7-Azidoazobenzene with NaHS. b) Absorbance spectra of 7-azidoazobenzene and aniline yellow. Aniline yellow does not react with NaHS while the azide reacts to form the amine, giving a yellow color. ($\lambda_{max}$ = 370 nm.)

4-Azidoazobenzene was synthesized in an analogous manner to AzMC and AzCC. When dissolved in buffer (with the aid of DMSO), 4-azidoazobenzene is pale yellow in color; however, addition of millimolar concentrations of NaHS results in the formation of a bright yellow solution. The absorbance spectra of this solution is identical to that of the parent 4-aminoazobenzene (aniline yellow) (Figure 5.9). Additionally, the yellow color shows a dose-dependent response to NaHS. While not as sensitive as the fluorescent probes or the colorimetric assay methylene blue, 4-azidoazobenzene is an example of a simple, colorimetric assay for hydrogen sulfide. It provides a rapid, visual

readout and does not require the addition of multiple, freshly prepared reagents, as in the methylene blue assay. In the future this approach could be optimized for sensitivity.

## 5.5 Conclusion

In conclusion, we have described a series of new and previously reported lanthanide complexes that exhibit $H_2S$-sensitive fluorescence. These complexes have significant utility in measuring $H_2S$ levels in samples from the petroleum industry. In particular, probe **3** has a detection limit of 250 nM NaHS in aqueous solutions, making it superior to both the methylene blue assay (with a detection limit around 10 μM $H_2S$)[21] and the industry standard ion selective electrode protocol (detection limit of 1.2 μM) in terms of sensitivity. Probe **1**, while less sensitive than probe **3**, has the advantage of a higher excitation wavelength and could be optimized for use in monitoring sulfide levels in crude oil samples. Finally, 4-azidoazobenze is an early example of a colorimetric hydrogen sulfide-sensitive probe.

## 5.6 References

(1) Szabó, C. *Nat. Rev. Drug Discov.* **2007**, *6*, 917–935.

(2) Srivastava, V. C. *RSC Adv.* **2012**, *2*, 759–783.

(3) Letterman, R. D. *Water Quality and Treatment: A Handbook of Community Water Supplies*; American Water Works Association, 1999; Vol. 331.

(4) Knight, L. D.; Presnell, S. E. *Am. J. Forensic Med. Pathol.* **2005**, *26*, 181–185.

(5) Fischer, E. *Berichte Dtsch. Chem. Gesellschaft* **1883**, *16*, 2234–2236.

(6) Caron, F.; Kramer, J. R. *Anal. Chem.* **1989**, *61*, 114–118.

(7) Hartman, M. C. T.; Dcona, M. M. *Analyst* **2013**, *137*, 4910–4912.

(8) Liu, T.; Xu, Z.; Spring, D. R.; Cui, J. *Org. Lett.* **2013**, *15*, 2310–2313.

(9) Galardon, E.; Tomas, A.; Roussel, P.; Artaud, I. *Dalt. Trans.* **2009**, 9126–9130.

(10) Lin, V. S.; Chang, C. J. *Curr. Opin. Chem. Biol.* **2012**, *16*, 595–601.

(11) Lippert, A. R.; New, E. J.; Chang, C. J. *J. Am. Chem. Soc.* **2011**, *133*, 10078–10080.

(12) Kolluru, G. K.; Shen, X.; Bir, S. C.; Kevil, C. G. *Nitric Oxide* **2013**, *35*, 5–20.

(13) Yuan, J.; Wang, G. *TrAC Trends Anal. Chem.* **2006**, *25*, 490–500.

(14) Muller, G. *Dalton Trans.* **2009**, 9692–9707.

(15) Zhang, H.; Hegde, A.; Ng, S. W.; Adhikari, S.; Moochhala, S. M.; Bhatia, M. *J. Immunol.* **2007**, *179*, 4153–4160.

(16) Zhang, H.; Zhi, L.; Moore, P. K.; Bhatia, M. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2006**, *290*, L1193–L1201.

(17) Zhang, J.; Sio, S. W. S.; Moochhala, S.; Bhatia, M. *Mol. Med.* **2010**, *16*, 417–424.

(18) Tropiano, M.; Faulkner, S. *Chem. Commun.* **2014**, *50*, 4696–4698.

(19) Coelho, A.; Castro, A. V; Dezotti, M.; Sant'Anna, G. L. *J. Hazard. Mater.* **2006**, *137*, 178–184.

(20) Thorson, M. K.; Majtan, T.; Kraus, J. P.; Barrios, A. M. *Angew. Chemie Int. Ed.* **2013**, *52*, 4641–4644.

(21) Ishigami, M.; Hiraki, K.; Umemura, K.; Ogasawara, Y.; Ishii, K.; Kimura, H. *Antioxid. Redox Signal.* **2009**, *11*, 205–214.

# APPENDIX A

# INHIBITION OF THE LYMPHOID TYROSINE PHOSPHATASE: THE EFFECT OF ZINC(II) IONS AND CHELATING LIGAND FRAGMENTS ON ENZYMATIC ACTIVITY*

## A.1 Abstract

A 96-member chelator fragment library (CFL-1.1) was screened to identify inhibitors of the lymphoid tyrosine phosphatase in the absence and presence of zinc acetate. Fragments that inhibit LYP activity more potently in the presence of zinc, fragments that rescue LYP activity in the presence of inhibitory concentrations of zinc, and fragments that inhibit LYP activity independent of zinc concentration were identified. Of these, 1,2-dihydroxynaphthalene was the most potent inhibitor with an $IC_{50}$ value of 2.52 ±0.06 μM after 2 h of incubation. LYP inhibition by 1,2-dihydroxy-naphthalene was very similar to inhibition by 1,2-naphthoquinone ($IC_{50}$ = 1.10 ±0.03), indicating that the oxidized quinone species is likely the active inhibitor. The inhibition was time-dependent, consistent with covalent modification of the enzyme.

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* This chapter is an adaptation of work accepted for publication.
Thorson, M. K.; Puerta, D. T.; Cohen, S. M.; Barrios, A. M. *Bioorg. Med. Chem. Lett.* **2014**.

## A.2 Introduction

The protein tyrosine phosphatases (PTPs) are a family of signaling enzymes that play critical roles in human health. For example, aberrant PTP activity has been implicated in diseases as diverse as cancer, metabolic disorders, and autoimmunity.[1–3] Reliant on a nucleophilic cysteine residue for activity, the PTPs are also susceptible to inhibition by heavy metals[4] and oxidation.[5,6] Because the PTPs play such instrumental roles in human biology, there is great interest in developing inhibitors that could serve as chemical probes for dissecting the biological roles of the PTPs as well as potential lead compounds for therapeutic development.[2,7,8]

One PTP of particular therapeutic interest is the lymphoid tyrosine phosphatase (LYP).[9] LYP serves as a negative regulator of early T cell receptor signaling and has been implicated in the development of autoimmunity.[10–12] Based on the known susceptibility of LYP to metal ions,[4] oxidizing agents,[5,6] and phosphotyrosine mimetic compounds such as salicylic acids,[13–16] we decided to undertake a small-scale, fragment-based screen to identify metal binding fragments that inhibit LYP activity either alone or in complex with metal ions. The chelator fragment library used in this work, CFL-1.1 (Figure A.1), incorporates a variety of metal-binding motifs in a total of 96 fragments.[17] Included in this library are phosphotyrosine mimetic moieties such as salicylic acids and picolinic acids and redox active fragments including catechols.

## A.3 Materials and Methods

*A.3.1 General considerations*. All chemicals were purchased from commercial sources unless indicated otherwise. $^{1}$H NMR data used for characterizing lead compounds

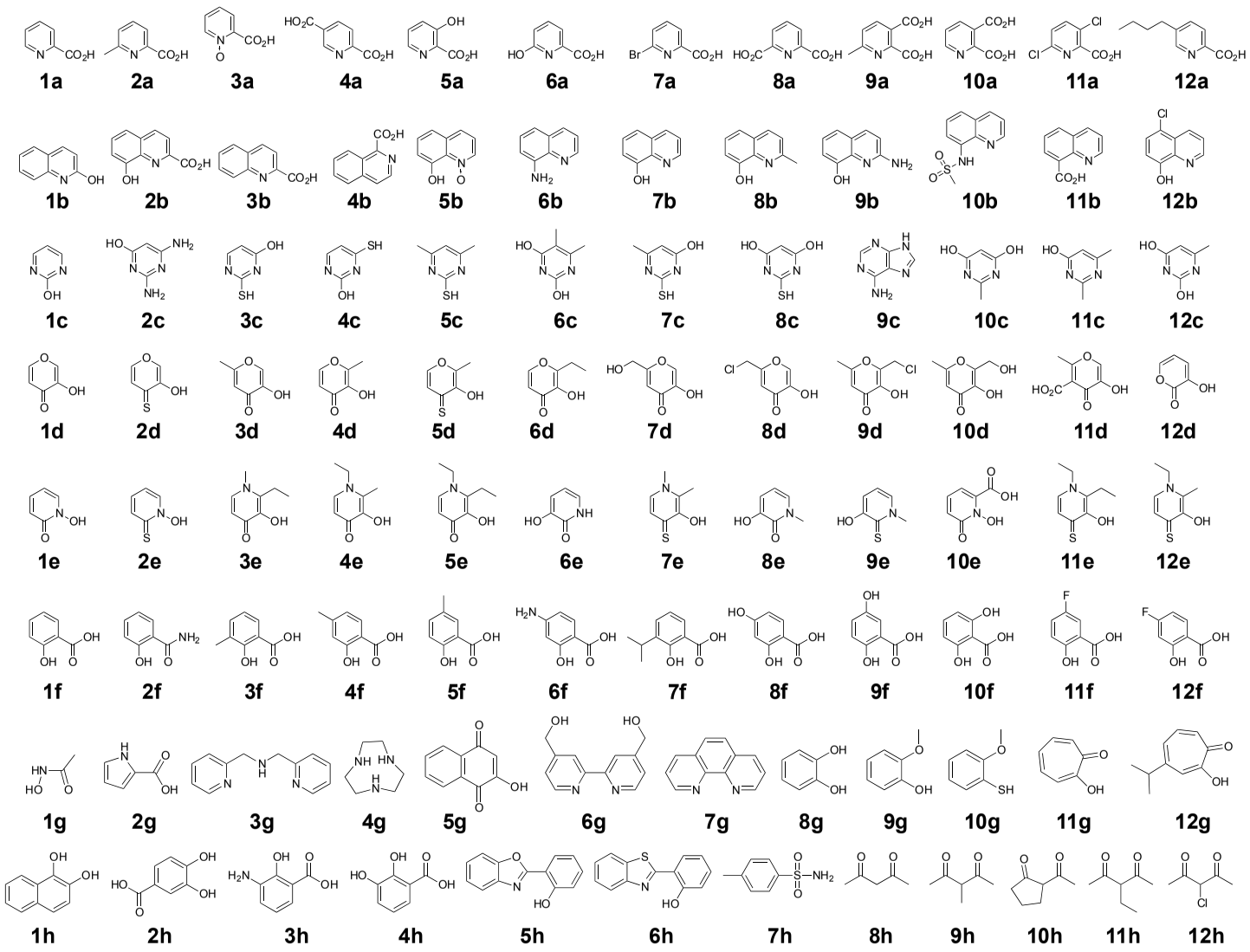


Figure A.1. Chelator fragment library, CFL-1.1

were collected on a Varian 400 MHz NMR spectrometer. Chemical shifts were measured in parts per million referenced to internal standard $(CH_3)_4Si$ = 0.00ppm. Fluorescence and UV/Vis were collected on a Molecular Devices Spectra Max M5. The catalytic domain of the lymphoid tyrosine phosphatase (LYP) was expressed and purified as previously described.[18,19]

*A.3.2 Zinc curves.* All studies were carried out in 94 µl buffer (50 mM Bis Tris, 100 mM NaCl, 0.01% Brij 35, pH 6.5) and 6 µl DMSO, which was used to dissolve the DiFMUP. The final probe concentration was 1.5 µM, and the final LYP concentration in each assay well was 5 nM. A 50 nM solution of LYP was activated with 1 mM TCEP for 30 min on ice prior to use, activating a new aliquot of LYP prior to each experiment. The activated enzyme was incubated with zinc for 30 min at 25 °C before DiFMUP was added to initiate the reaction.

*A.3.3 Library screen.* As with the zinc curves, all studies were carried out in 94 µl buffer (50 mM Bis Tris, 100 mM NaCl, 0.01% Brij 35, pH 6.5) and 6 µl DMSO, which was used to dissolve both the DiFMUP and the compounds. The final probe concentration was 1.5 µM, the final LYP concentration was 5 nM, and the final concentration of each library compound was 50 µM. As above, LYP was preactivated with TCEP prior to use. The compound, LYP, and zinc (if using) were incubated for 30 min at 25 °C before starting the reaction with the addition of DiFMUP.

*A.3.4 Time-dependent inhibition of LYP by 1,2-dihyrdoxynaphthalene.* The total DMSO concentration was held at 6%. In 100 µL total reaction volume (buffer: 50 mM Bis-Tris, 100mM NaCl, 0.01% Brij 35, 2mM EDTA, pH 6.5) 1,2-Dihyroxy-naphthalene was mixed with buffer with LYP progressively over 2 hours. The LYP was activated as

described and stored on ice. Immediately after the last addition (incubation time = "0 min."), DiFMUP was added to start the experiment.

*A.3.5 Selectivity of 1,2-Dihydroxynaphthalene for LYP over PTP1B, CD45, and YOP*. LYP, PTP1B, YOP and CD45 were activated and used as described above and all used at a final concentration of 5 nM in 50 mM Bis-Tris, 100 mM NaCl, 0.01% Brij 35, 2 mM EDTA, pH 6.5 buffer.

**A.4 Discussion of Results**

Initial investigations into the effect of zinc(II) on LYP activity under our standard assay conditions demonstrated that zinc is an effective inhibitor of LYP, with complete inhibition achieved in the presence of 100 μM zinc(II) acetate. This is not surprising, as thiophilic metal ions have been shown to act as competitive, pseudo-irreversible inhibitors of PTP activity, interacting with the catalytic cysteine residue.[4,20–22] As shown in Figure A.2, in the presence of 40 μM of zinc acetate, the LYP activity was reduced to 20% of the control, facilitating the identification of chelators that may rescue zinc-mediated enzyme inhibition by binding to and removing the zinc from the enzyme active site. At 5 μM zinc acetate, the activity of LYP was reduced to 80% of the control, providing a useful starting point from which to identify chelators that may act synergistically with zinc to inhibit LYP activity. Using the information from the initial dose-response data with zinc acetate, three separate screens of the fragment library CFL-1.1 were carried out: (1) in the presence of 40 μM zinc acetate to identify chelators capable of removing zinc from the active site of LYP and rescuing the enzyme from zinc-mediated inhibition, (2) in the presence of 5 μM zinc acetate in order to identify

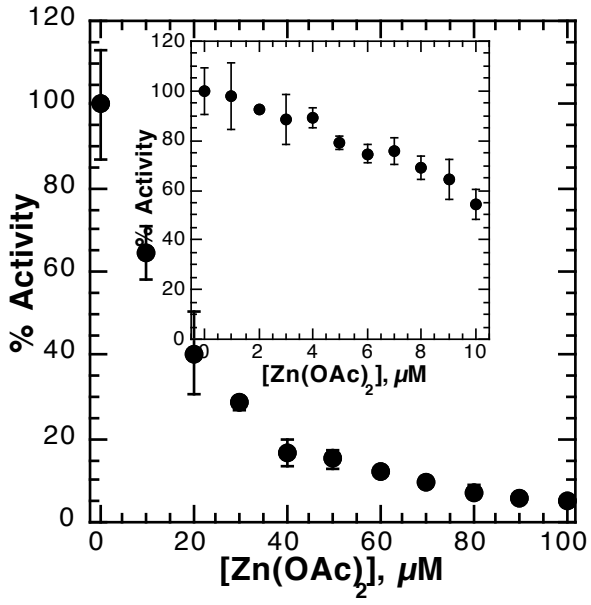


Figure A.2. Inhibition of LYP activity by zinc acetate. Enzyme activity (defined as 100% in the absence of Zn) decreases in a dose dependent manner as $Zn(OAc)_2$ is added, with complete inhibition achieved at 100 μM added Zn(II). Inset shows the response to low concentrations of Zn(II).

compounds that display enhanced inhibition in the presence of zinc, and (3) in the absence of zinc in order to identify fragments capable of inhibiting LYP activity on their own.

As indicated in Figure A.3, di-(2-picolyl)-amine (**3g**), 5-chloro-8-quinolol (**12b**), and 2,6-pyridine dicarboxylic acid (**8a**) had little effect on enzymatic activity alone, but were each capable of rescuing the enzyme from zinc-mediated inhibition. These compounds are all known zinc chelators, and their ability to restore enzyme activity in the presence of zinc is consistent with the hypothesis that they may sequester zinc, removing it from the enzyme active site. The observation that the chelators are able to activate LYP slightly in the absence of added zinc is consistent with the sensitivity of the enzyme to inhibition by adventitious metal. Indeed, tyrosine phosphatase assays are

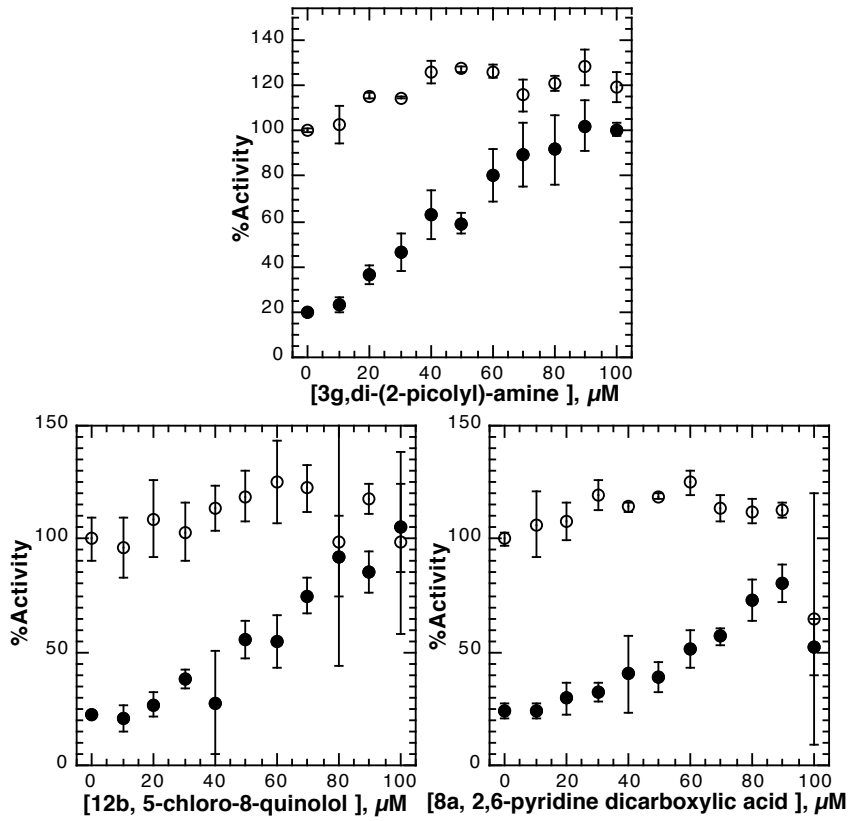


Figure A.3. Compounds 3g, 12b, and 8a have little effect on LYP activity on their own (open circles) but are all capable of restoring LYP activity in the presence of 40 μM zinc acetate (solid circles). Two equiv of chelator (80 μM) is sufficient to restore activity in the case of 3g and 12b, with slightly more than two equiv required for 8a.

usually carried out in a buffer containing EDTA to avoid this problem.[23] It appears that, under the conditions of this assay, approximately two equivalents of each chelator (relative to zinc) are required to restore full activity.

A handful of compounds showed the potential for chelator-enhanced inhibition in the presence of 5 μM zinc acetate in our initial screen. Of these, only compound **5g** (2-hydroxy-1,4-naphthoquinone) showed consistent concentration-dependent inhibition of LYP in the presence of zinc (Figure A.4) in follow-up studies. The interpretation of these results is ambiguous because 2-hydroxy-1,4-naphthoquinone is a Michael acceptor.

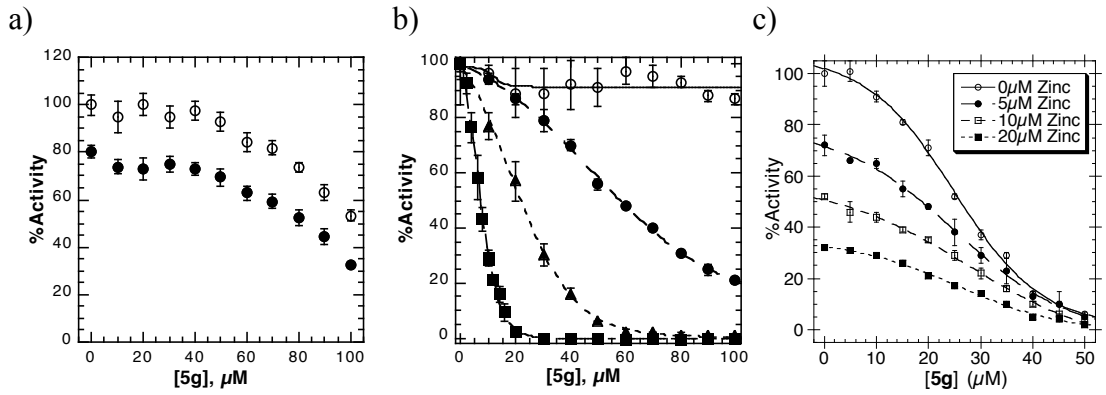


Figure A.4. Inhibition of LYP by compound 5g. a) Compound **5g** inhibits LYP activity more potently in the presence of zinc (solid circles) than in the absence of zinc (open circles). b) Compound **5g** is a time-dependent inhibitor of LYP activity, showing increased inhibition as incubation time increases from 0 min (open circles) to 30 min (solid circles) to 60 min (triangles) and finally 120 min (squares). c) Inhibition of LYP by compound **5g** in the presence of increasing amounts of zinc: 0 μM (open circles), 5 μM (closed circles), 10 μM (open squares), 20 μM (closed squares).

Similar compounds have been shown to form pseudo-irreversible adducts with the catalytic cysteine residue.[24] Indeed, in the absence of zinc, compound **5g** showed time-dependent inhibition, with essentially no inhibition at the initial time point but an $IC_{50}$ value of 5.6 ±0.3 μM after 2 h of incubation. In the presence of zinc, there appears to be an additive effect in the inhibition of LYP by compound **5g** (Figure A.4c).

The most potent hit from CFL-1.1 was compound **1h**, 1,2-dihydroxynaphthalene. Compound **1h** was also a time-dependent inhibitor of LYP activity, with an $IC_{50}$ of 2.52 ±0.06 μM after 2 h of incubation (Figure A.5). Compounds related to 1,2-dihydroxy-naphthalene have been reported as PTP inhibitors previously, although it is likely the oxidized version of this compound that is active.[25] In the presence of oxygen, **1h** is readily oxidized to 1,2-naphthoquinone, an oxidant that has been reported to inhibit PTP

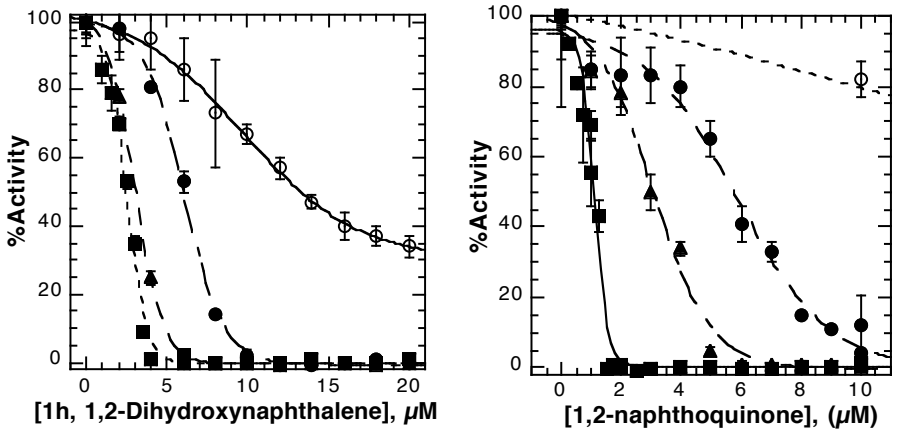


Figure A.5. Compound 1h (left panel) and 1,2-naphthoquinone (right panel) inhibit LYP activity in a time-dependent manner, showing increased inhibition as incubation time increases from 0 min (open circles) to 30 min (solid circles) to 60 min (triangles) and finally 120 min (squares).

activity.[26] Indeed, 1,2-naphthoquinone shows very similar activity against LYP (Figure A.5), with an $IC_{50}$ value of 1.10 ±0.03 μM after 2 h of incubation. As mentioned previously, similar compounds have been shown to form pseudo-irreversible adducts with the catalytic cysteine residue of the PTPs.[24] However, 1,4-naphthoquinone derivatives have also been reported as selective allosteric inhibitors of PTP activity, acting through a non-redox-mediated mechanism.[27] Interestingly, compound **1h** has modest selectivity for LYP over PTP1B, CD45, and YopH (Figure A.6). This selectivity is not dramatic, but could serve as the starting point for building a more potent and selective LYP inhibitor.

## A.5 Conclusion

From a library of 96 metal-binding fragments, we have identified a series of inhibitors of the lymphoid tyrosine phosphatase, LYP. The most potent inhibitors were

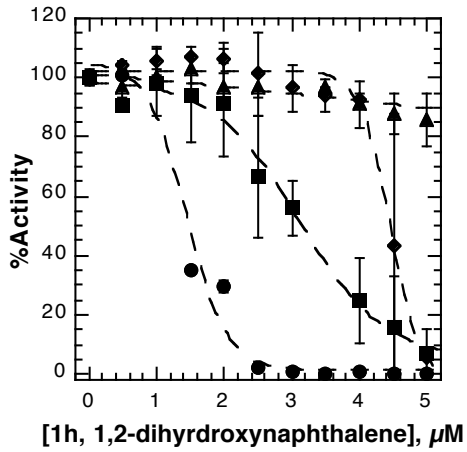


Figure A.6. Inhibition of LYP (circles) CD45 (squares), PTP1B (diamonds) and YopH (triangles) by compound 1h, 1,2-dihydroxynaphthalene.

the naphthoquinones **5g** and **1h**, which displayed time-dependent LYP inhibition consistent with covalent adduct. The 1,2-dihydroxynaphthalene scaffold has modest inherent selectivity for LYP over a handful of other PTPs.

**A.6 References**

(1) Alonso, A.; Sasin, J.; Bottini, N.; Friedberg, I.; Friedberg, I.; Osterman, A.; Godzik, A.; Hunter, T.; Dixon, J.; Mustelin, T. *Cell* **2004**, *117*, 699–711.

(2) Bialy, L.; Waldmann, H. *Angew. Chem. Int. Ed. Engl.* **2005**, *44*, 3814–3839.

(3) Zhang, Z. Y. *Curr. Opin. Chem. Biol.* **2001**, *5*, 416–423.

(4) Lu, L.; Zhu, M. *Antioxid. Redox Signal.* **2014**, *20*, 2210–2224.

(5) Karisch, R.; Fernandez, M.; Taylor, P.; Virtanen, C.; St-Germain, J. R.; Jin, L. L.; Harris, I. S.; Mori, J.; Mak, T. W.; Senis, Y. A.; Östman, A.; Moran, M. F.; Neel, B. G. *Cell* **2011**, *146*, 826–840.

(6) Tonks, N. K. *Cell* **2005**, *121*, 667–670.

(7) Blaskovich, M. A. T. *Curr. Med. Chem.* **2009**, *16*, 2095–2176.

(8) Heneberg, P. *Curr. Med. Chem.* **2009**, *16*, 706–733.

(9) Cohen, S.; Dadi, H.; Shaoul, E.; Sharfe, N.; Roifman, C. M. *Blood* **1999**, *93*, 2013–2024.

(10) Bottini, N.; Musumeci, L.; Alonso, A.; Rahmouni, S.; Nika, K.; Rostamkhani, M.; MacMurray, J.; Meloni, G. F.; Lucarelli, P.; Pellecchia, M.; Eisenbarth, G. S.; Comings, D.; Mustelin, T. *Nat. Genet.* **2004**, *36*, 337–338.

(11) Carlton, V. E. H.; Hu, X.; Chokkalingam, A. P.; Schrodi, S. J.; Brandon, R.; Alexander, H. C.; Chang, M.; Catanese, J. J.; Leong, D. U.; Ardlie, K. G.; Kastner, D. L.; Seldin, M. F.; Criswell, L. A.; Gregersen, P. K.; Beasley, E.; Thomson, G.; Amos, C. I.; Begovich, A. B. *Am. J. Hum. Genet.* **2005**, *77*, 567–581.

(12) Vang, T.; Miletic, A. V; Arimura, Y.; Tautz, L.; Rickert, R. C.; Mustelin, T. *Annu. Rev. Immunol.* **2008**, *26*, 29–55.

(13) He, Y.; Liu, S.; Menon, A.; Stanford, S.; Oppong, E.; Gunawan, A. M.; Wu, L.; Wu, D. J.; Barrios, A. M.; Bottini, N.; Cato, A. C. B.; Zhang, Z.-Y. *J. Med. Chem.* **2013**, *56*, 4990–5008.

(14) Vang, T.; Xie, Y.; Liu, W. H.; Vidović, D.; Liu, Y.; Wu, S.; Smith, D. H.; Rinderspacher, A.; Chung, C.; Gong, G.; Mustelin, T.; Landry, D. W.; Rickert, R. C.; Schürer, S. C.; Deng, S.-X.; Tautz, L. *J. Med. Chem.* **2011**, *54*, 562–571.

(15) Xie, Y.; Liu, Y.; Gong, G.; Rinderspacher, A.; Deng, S.-X.; Smith, D. H.; Toebben, U.; Tzilianos, E.; Branden, L.; Vidović, D.; Chung, C.; Schürer, S.; Tautz, L.; Landry, D. W. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2840–2844.

(16) Yu, X.; Sun, J.-P.; He, Y.; Guo, X.; Liu, S.; Zhou, B.; Hudmon, A.; Zhang, Z.-Y. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 19767–19772.

(17) Jacobsen, J. A.; Fullagar, J. L.; Miller, M. T.; Cohen, S. M. *J. Med. Chem.* **2011**, *54*, 591–602.

(18) Tsai, S. J.; Sen, U.; Zhao, L.; Greenleaf, W. B.; Dasgupta, J.; Fiorillo, E.; Orrú, V.; Bottini, N.; Chen, X. S. *Biochemistry* **2009**, *48*, 4838–4845.

(19) Ahmed, V. F.; Bottini, N.; Barrios, A. M. *ChemMedChem* **2014**, *9*, 296–299.

(20) Karver, M. R.; Krishnamurthy, D.; Bottini, N.; Barrios, A. M. *J. Inorg. Biochem.* **2010**, *104*, 268–273.

(21) Karver, M. R.; Krishnamurthy, D.; Kulkarni, R. A.; Bottini, N.; Barrios, A. M. *J. Med. Chem.* **2009**, *52*, 6912–6918.

(22) Wilson, M.; Hogstrand, C.; Maret, W. *J. Biol. Chem.* **2012**, *287*, 9322–9326.

(23) Montalibet, J.; Skorey, K. I.; Kennedy, B. P. *Methods* **2005**, *35*, 2–8.

(24) Iwamoto, N.; Sumi, D.; Ishii, T.; Uchida, K.; Cho, A. K.; Froines, J. R.; Kumagai, Y. *J. Biol. Chem.* **2007**, *282*, 33396–33404.

(25) Ahn, J.; Cho, S.; Ha, J.; Kang, S.; Jung, S.; Kim, H.-M.; Kim, S.; Kim, K.; Cheon, H.; Yang, S.-D.; Choi, J.-K. *Bull. Korean Chem. Soc.* **2003**, *24*, 1505–1508.

(26) Phillips, R. M.; Bair, E.; Lawrence, D. S.; Sims, C. E.; Allbritton, N. L. *Anal. Chem.* **2013**, *85*, 6136–6142.

(27) Perron, M. D.; Chowdhury, S.; Aubry, I.; Purisima, E.; Tremblay, M. L.; Saragovi, H. U. *Mol. Pharmacol.* **2014**, *85*, 553–563.